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Nutrition and bone metabolism:
In vivo effects of inorganic phosphate on rats and in vitro effects of bioactive tripeptides on human osteoblasts

ACADEMIC DISSERTATION

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‘Scientists don’t set out to make discoveries,
they set out to uncover stories.’

Len Fisher

CONTENTS

ABSTRACT

Nutrition affects bone health throughout life. To optimize peak bone mass development and maintenance, it is important to pay attention to the dietary factors that enhance and impair bone metabolism. In this study, the in vivo effects of inorganic dietary phosphate and the in vitro effects of bioactive tripeptides, IPP, VPP and LKP were investigated.

Dietary phosphate intake is increased through the use of convenience foods and soft drinks rich in phosphate-containing food additives. Our results show that increased dietary phosphate intake hinders mineral deposition in cortical bone and diminishes bone mineral density (BMD) in the aged skeleton in a rodent model (Study I). Phosphate elevates parathyroid hormone (PTH) concentration and conserves trabecular bone, as mineral loss was lower in the lumbar spine with a phosphate-rich diet. In the growing skeleton (Study II), increased phosphate intake was observed to reduce bone material and structural properties, leading to diminished bone strength. Studies I and II revealed that a low Ca:P ratio has negative effects on the mature and growing rat skeleton even when calcium intake is sufficient.

High dietary protein intake is beneficial for bone health. Protein is essential for bone turnover and matrix formation. In addition, hydrolysis of proteins in the gastrointestinal tract produces short peptides that possess a biological function beyond that of being tissue building blocks. The effects of three bioactive tripeptides, IPP, VPP and LKP, were assessed in short- and long-term in vitro experiments. Short-term treatment (24 h) with tripeptide IPP, VPP or LKP influenced osteoblast gene expression (Study III). IPP in particular, regulates genes associated with cell differentiation, cell growth and cell signal transduction. The upregulation of these genes indicates that IPP enhances osteoblast proliferation and differentiation. IPP was confirmed to increase UMR-106 and hMSC proliferation, but not the proliferation of mature osteoblasts. Long-term treatment with IPP enhanced osteoblast gene expression in favour of bone formation and increased mineralization (Study IV), but did not influence osteoblast differentiation in vitro. The in vivo effects of IPP on osteoblast differentiation might differ since eating frequency drives food consumption, and protein degradation products, such as bioactive peptides, are available periodically, not continuously as in this study.

To sum up, Studies I and II raise concern about the appropriate amount of dietary phosphate to support bone health as excess is harmful. Studies III and IV in turn, support findings of the beneficial effects of dietary protein on bone and provide a mechanistic explanation since cell proliferation and osteoblast function were improved by treatment with bioactive tripeptide IPP.

ABBREVIATIONS

ACE, angiotensin-I-converting enzyme
ALP, alkaline phosphatase
Apaf, apoptotic protease-activating factor
BMD, bone mineral density
BMP, bone morphogenetic protein
BMU, basic multicellular units
cAMP, cyclic adenosinmonophosphate
CaR, calcium-sensing receptor
Cbfa1/Runx2, core binding factor alpha 1/runt-related gene 2
CREB-5, cAMP response element binding protein 5
Dkk, Dickkopf homologue
ECM, extracellular matrix
FADD, Fas-associated death domain
FGF, fibroblast growth factor
Fz, frizzled receptor
GIP, gastric inhibitory polypeptide
GLP, glucagon-like peptide
Hh, hedgehog
hPepT, human di/tri-peptide transporter
IGF, insulin-like growth factor
Ihh, Indian hedgehog
IPP, Ile-Pro-Pro
LKP, Leu-Lys-Pro
LRP low-density lipoprotein receptor-related protein
M-CSF, macrophage colony-stimulating factor
NF-κB, nuclear factor kappa B
Npt, Na/Pi co-transporter
OCN, osteocalcin
OPG, osteoprotegerin
Osx, osterix
OVX, ovarectomy
PBM, peak bone mass
Pi, inorganic phosphate
PKA, protein kinase A
PTH, parathyroid hormone
PTHrP, parathyroid hormone-related peptide
RANKL, receptor activator of nuclear factor kappa B ligand
RGD, Arg-Gly-Asp
TGF, transforming growth factor
VPP, Val-Pro-Pro

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals:

I Huttunen MM. Pietilä PE. Viljakainen HT. Lamberg-Allardt CJE. Prolonged increase in dietary phosphate intake alters bone mineralization in adult male rats. J Nutr Biochem 2006;17(7):479-484

II Huttunen MM. Tillman I. Viljakainen HT. Tuukkanen J. Peng Z. Pekkinen M. Lamberg-Allardt CJE. High dietary phosphate intake reduces bone strength in the growing rat skeleton. J Bone Miner Res 2007;22(1):83-92

III Huttunen MM. Pekkinen M. Ahlström M. Lamberg-Allardt CJE. The effects of bioactive tripeptides IPP (Ile-Pro-Pro), VPP (Val-Pro-Pro) and LKP (Leu-Lys-Pro) on gene expression of osteoblasts differentiated from human mesenchymal stem cells. Br J Nutr, accepted

IV Huttunen MM. Pekkinen M. Ahlström M. Lamberg-Allardt CJE. Long-term effects of tripeptide IPP on osteoblast differentiation in vitro. J Nutr Biochem, conditionally accepted

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1 INTRODUCTION

The World Osteoporosis Day on the 20th of October 2006 was themed 'Bone appetite' to focus on the role of nutrition in building bones. Osteoporosis is a disease characterized by brittle and porous bones that are prone to fracture. The disease affects about 75 million people in Europe, USA and Japan. The International Osteoporosis Foundation estimated the total cost of osteoporotic fractures to be € 31.7 billion in Europe in 2002. As the population ages, the number of individuals with osteoporosis will increase and the cost incurred by society will, in turn, rise.

Prevention is the most cost-effective means of tackling the osteoporosis issue. Bone mass attained early in life may be the most important determinant of lifelong bone health (NIH consensus conference 2001). Genetic factors play a predominant role in peak bone mass (PBM) development, but physiological, environmental and modifiable lifestyle factors can also have a significant role. A balanced diet, adequate energy and nutrient intake lay the foundation for all tissue development, including bone. Calcium is the most important nutrient for attaining PBM and for preventing bone loss. Several vitamins, such as vitamin D (calcium absorption), and vitamins A, K, and E (bone matrix synthesis), are associated with bone health. Other nutritional aspects, such as eating patterns (consumption of fruits and vegetables boosts bone health) and eating frequencies (frequent meals enhance bone health, likely through gastrointestinal hormone secretion), also affect bone health. One goal for nutritional research in the bone field is to establish the optimal diet to enable maximal PBM development and to maintain the bone mass achieved. Nutrients important for bone should be recommended, but bone-impairing nutrients must also be elucidated.

This thesis is composed of in vivo (Studies I and II) and in vitro (Studies III and IV) studies exploring the effects of inorganic dietary phosphate and bioactive peptides on bone metabolism. The work contributes to a better understanding of the mechanisms of action of phosphate and bioactive peptides on bone at the tissue and cellular level.

2 REVIEW OF THE LITERATURE

2.1 Structure and function of bone

Bone tissue consists of an organic matrix, minerals and bone cells. A major organic component is type I collagen, but other proteins, such as osteocalcin, osteonectin and osteopontin, are also present. The main mineral in bone is hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), found in conjunction with carbonate, citrate, magnesium, sodium, fluoride and strontium ions. Bone cells comprise a population of cells of heterogeneous origin and restricted function with respect to matrix formation, mineralization and resorption. The local mesenchymal origin of bone-forming osteoblast cells differs from that of extraskeletal, hemopoietic bone-resorbing osteoclast relatives. However, the functions of these two diverse populations are strongly related and interdependent.

The bone formed through the ossification process can be divided into compact cortical bone and spongiosa trabecular bone. Trabecular bone forms 20% of bone mass, but its high remodelling rate renders its metabolic activity equal to that of cortical bone (Eriksen et al. 1994). Bone remodelling is a surface phenomena and because trabecular bone has more surface area than cortical bone, also the remodelling rate is higher (Arnett 2003). Furthermore, trabecular and cortical bone have different functions. Trabecular bone has more of a "metabolic" function in quickly releasing mineral ions into blood and cortical bone has more of a mechanical and protective function.

2.1.1 Osteoblasts

The osteoblast lineage consists of osteoblasts, osteocytes and bone-lining cells. Osteoblasts are bone-forming cells that synthesize and secrete most of the proteins of the bone extracellular matrix (ECM) and also express the genes necessary and sufficient to induce mineralization. During bone formation some osteoblasts become entrapped in the mineralized matrix and differentiate further into osteocytes. Approximately one in 12 osteoblasts undergo this change (Noble 2003). Osteocytes are the most abundant cell type in bone, outnumbering osteoblasts by a factor of ten (Manolagas 2000). They have a stellate morphology and they form a dentritic network throughout the mineralized matrix. Osteocytes use this network to communicate with each other and the cells on the bone surface. Osteocytes function as the mechanosensors to start bone remodelling in order to adapt bone architecture to the mechanical needs of the skeleton (Noble & Reeve 2000). Evidence also indicates that osteocyte apoptosis attracts osteoclasts to damaged bone areas to initiate the remodelling process (Noble et al. 1997). Intact osteocyte network inhibits osteoclast activity by secreted signal molecules but if the network is damaged inhibition ceases and osteoclasts attach to the bone to start resorption (Gu et al. 2005). Instead of differentiating into osteocytes, some osteoblasts become bone-lining cells. These are flat, elongated cells on top of the unmineralized collagen matrix. Evidence suggests that lining cells are capable of reverting back to osteoblasts (Bellido et al. 2003), but they are also thought to be the target cells of osteocyte signalling (Manolagas 2000, Noble & Reeve 2000). In response to osteocyte signals,

lining cells are likely to be the ones prepairing the bone surface for osteoclast attachment (Manolagas 2000).

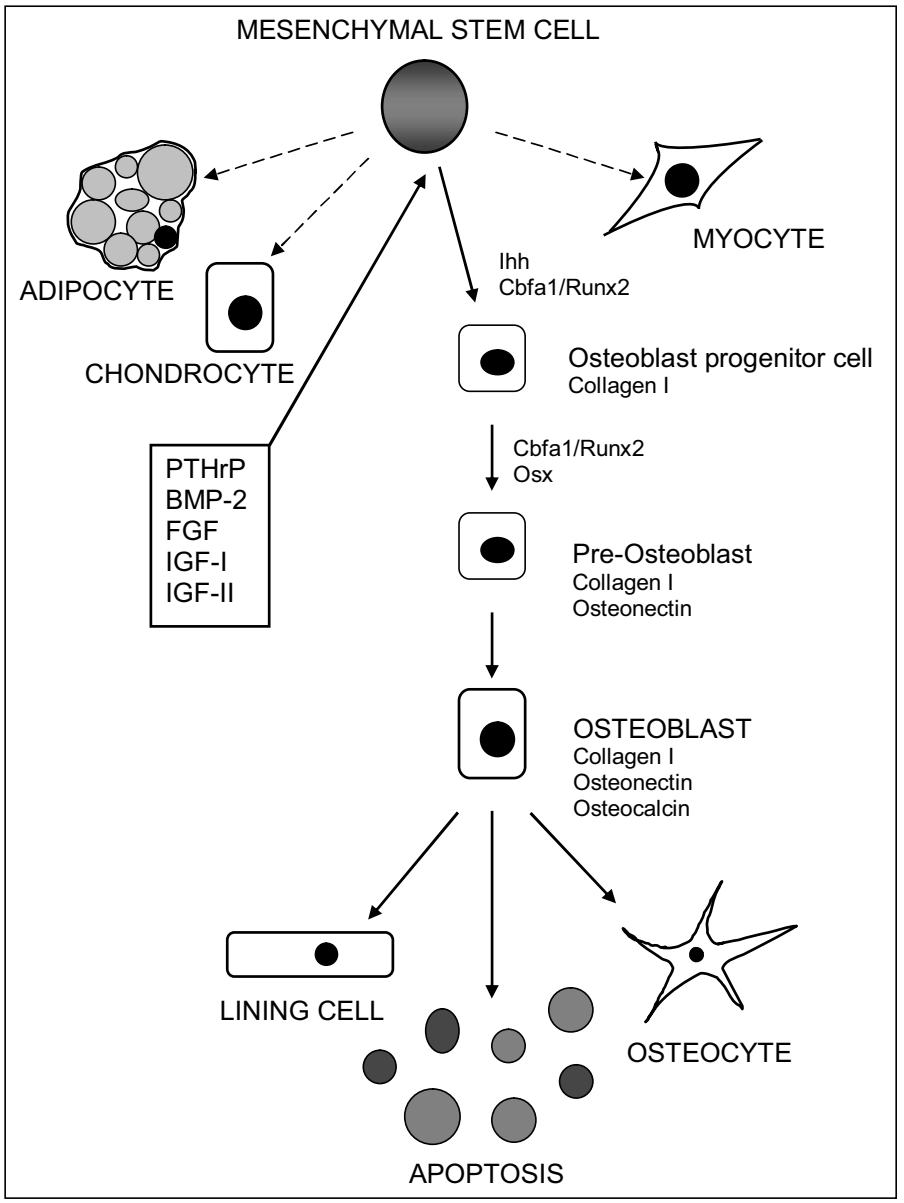

Figure 1: Bone marrow-derived mesenchymal stem cells are capable of differentiating into adipocytes, chondrocytes, osteoblasts, and myocytes. Osteoblast differentiation is potentiated with factors such as PTHrP, BMP-2, FGF, IGF-I and –II. The first gene associated with osteoblast differentiation is Ihh, followed by Cbfa1/Runx2. Osteoblast progenitor cell expresses collagen type I. Osterix (Osx) gene is expressed downstream of Cbfa1/Runx2 in pre-osteoblasts. Osx promotes osteonectin expression. In mature osteoblasts the expression of

non-collagenous proteins, such as osteocalcin is further increased. After completing bone-forming function osteoblast can turn into bone lining cell or osteocyte, but the majority of cells die of apoptosis.

2.1.1.1 Osteoblast differentiation

The same pluripotent mesenchymal stem cells that differentiate into chondrocytes during endochondral ossification are also capable of differentiating into myoblasts, adipocytes and osteoblasts (Figure 1). The osteoblast differentiation process is regulated by hormones and cytokines that activate osteoblast-specific signalling proteins and transcription factors.

There are at least two osteogenic pathways: hedgehog (Hh) and Wnt. The Hh signalling pathway influences transcription of many genes (for review, see Hooper & Scott 2005). The three Hh genes in mammals, sonic (Ssh), Indian (Ihh) and desert hedgehog (Dhh), are expressed in different tissues and at different stages of development, and might vary in biological activity. During endochondral ossification the pre-chondrocytes in the cartilage-forming model express Ihh, which plays a role in coordinating the growth and differentiation of chondrocytes (Mak et al. 2006, Rodda & McMahon 2006). In addition, Ihh initiates the osteogenic programme in osteoblast progenitors and is required for osteoblast development in vivo (Long et al. 2004). Hh signalling acts at the early stages of osteoblast differentiation, activating the expression of Cbfa1/Runx2, collagen 1 and alkaline phosphatase (Hu et al. 2004).

Core binding factor alpha 1 (Cbfa1), also called runt-related gene 2 (Runx2), is the most extensively studied osteogenic transcription factor. Cbfa1/Runx2 is necessary for osteogenesis. Cbfa1/Runx2 activates osteocalcin, an osteoblast-specific gene expressed by fully differentiated osteoblasts (Ducy 2000). Cbfa1/Runx2-deficient mice lack bone formation because of a maturation arrest of osteoblasts (Komori et al.1997). However, Cbfa1/Runx2 alone is not sufficient for osteogenesis. Thus, besides Cbfa1/Runx2, other transcription factors are needed to activate the genetic pathways controlling osteoblast differentiation.

In addition to initiating osteoblastogenesis, Hh induces the expression of Wnt ligands. The Wnt proteins are a family of 19 secreted signalling molecules (Kuhl 2004). Wnts are important in osteoblast differentiation. The Wnt/β-catenin canonical pathway is activated when Wnt binds to the frizzled receptor (Fz) and the low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) co-receptor located on the cell membrane (Figure 2). The signals generated stabilize β-catenin and the accumulated protein is then translocated to the nucleus, where it functions as a transcriptional co-activator (Krishnan et al. 2006).

The Wnt/β-catenin pathway regulates bone development through different mechanisms during different stages of life (Krishnan et al. 2006, Rodda & McMahon 2006). In the early stages of osteoblastogenesis, the Wnt/β-catenin pathway stimulates Cbfa1/Runx2 expression (Gaur et al. 2005), and in the later stages it downregulates RANKL expression (Spencer et al. 2006). In

addition to stimulating osteoblastogenesis and reducing osteoclastogenesis, the Wnt/β-catenin pathway represses adipogenesis (Bennett et al. 2002, Arango et al. 2005) and protects against apoptosis (Day et al. 2005, Krishnan et al. 2006). An interesting aspect of the Wnt/β-catenin pathway is that mechanical stimulation enhances bone mass through LRP5 signalling and causes β-catenin to translocate to the nucleus (Robinson et al. 2006, Sawakami et al. 2006). Furthermore, LRP5 appears to be a potent regulator of bone mass, size and strength (Sawakami et al. 2006). Wnt signalling can be downregulated by Dickkopf homologue 1 (Dkk1). The inhibiton of Wnt signalling and the removal of β-catenin prevents the maturation of osteoblasts (Hu et al. 2004, Rodda & McMahon 2006). While canonical Wnt signalling itself is not sufficient to promote osteoblast differentiation, it can synergize with bone morphogenetic protein (BMP) signalling (Day et al. 2005).

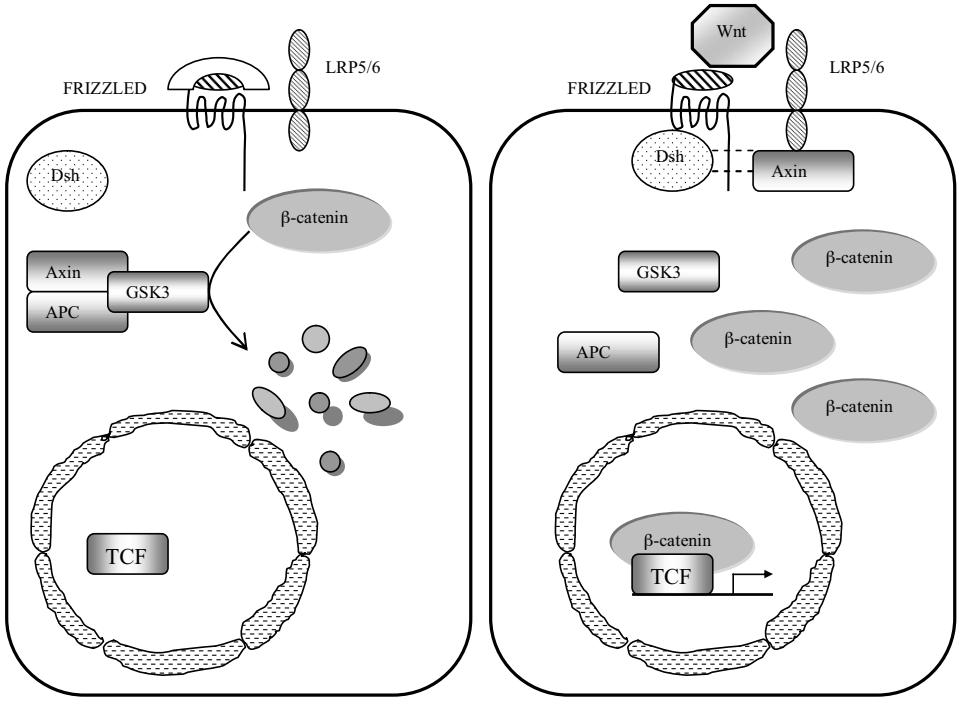

Figure 2: The canonical Wnt signalling pathway. The left figure shows the degradation of β-catenin through interaction with Axin, APC and glycogen synthase kinase-3 (GSK3). Dickkopf homologue 1 (Dkk1) prevents Wnt from binding to the Frizzled receptor. Right figure shows the binding of Wnt protein to the Frizzled/LRP5/6 co-receptor complex. The activation of Dishevelled (Dsh) causes Axin to be recruited to the membrane and interact with LRP5/6. The inactivation of GSK3 results in the stabilization and accumulation of β-catenin in the cytoplasm and nucleus. In the nucleus, β-catenin interacts with T-cell factor/lymphoid enhancer binding factor (TCF) and activates transcription of Wnt/β-catenin target genes (Logan & Nusse 2004).

BMPs are members of the transforming growth factor-β (TGF-β) superfamily, and their fundamental function is to induce differentiation of mesenchymal cells into cells of the osteoblastic lineage (Canalis et al. 2003). As a result, the pool of mature osteoblastic cells increases (Canalis et al. 2003). The BMP family can be divided into several subgroups based on BMP gene sequence identity. In osteoblasts, BMPs regulate growth, differentiation and apoptosis. BMP-2, BMP-4 and BMP-7 upregulate Cbfa1/Runx2 expression, inducing osteoblast differentiation (Yamaguchi et al. 2000, Phimphilai et al. 2006). BMP-6 induces osteoblast differentiation and maturation (Ebisawa et al. 1999) and is involved in in vitro bone nodule formation (Yamaguchi et al. 2000). In an ovarectomy (OVX) rodent model, BMP-6 increased bone volume, enhanced bone microarchitecture and restored bone quality (Simic et al. 2006). BMP-5 is closely related to BMP-6, as they both belong to the same subgroup of the BMP family tree. Hence, BMP-5 has similar osteoinductive properties (Wozney 2002). BMP-5 plays a role in fracture healing, and one characteristic of BMP-5 null mice is impaired fracture repair (Canalis et al. 2003).

BMPs and Wnts are important signals in osteoblast differentiation. Notch is a signaling pathway opposingWnt/β-catenin signaling (Deregowski et al. 2005, Zamurovic et al. 2004). Notch proteins are transmembrane receptors that play a critical role to control cell fates. The Notch signaling pathway is activated through direct cell–cell contact of the Notch receptor with one of its ligands. Notch interaction with Wnt signaling decreases β-catenin and prevents the transcription of Wnt target genes (Deregowski et al. 2005). Notch ligand Jagged1 plays an important role in MSC differentiation. In osteoblasts Jagged1 is regulated by PTH through the activation of the AC/PKA pathway (Weber et al. 2006). Jagged1/Notch signaling modulates osteoblastic differentiation but there is controversy wether it is impaired (Sciaudone et al. 2002) or enhanced (Tezuka et al. 2002).

During in vitro differentiation from an osteoprogenitor to a mature osteoblast, three distinct phases can be distinguished that reflect the maturation stages of osteoblasts in vivo (Dworetzky et al. 1990): proliferation, matrix maturation and mineralization. Different markers have been associated with each of these phases. Markers are useful since in a cell culture osteoblasts are nearly indistinguishable from fibroblasts and their only specific morphological feature is the capability to form mineralized nodules (Ducy et al. 2000). At the proliferation phase, cell growth and cell cycle genes are actively expressed, as are type I collagen genes (Owen et al. 1990). At the matrix maturation phase, alkaline phosphatase (ALP) and osteopontin gene expression occurs. Osteoblast cell proliferation becomes limited as the state of differentiation increases (Owen et al. 1990, Amédée et al. 1994, Qi et al. 2003). Proliferation and differentiation are generally considered to be opposite and mutually exclusive states in cell biology (Selz et al. 1989). At the onset of mineralization, the expression of non-collagenous proteins (osteopontin and osteocalcin) is further increased, and calcium and phosphate are deposited into the ECM (Owen et al.1990). OPG expression is also increased at the onset of mineralization, remaining high in mature osteoblasts and resulting in a lower RANKL/OPG ratio and suppression of osteoclast differentiation (Thomas et al. 2001).

2.1.1.2 Bone formation

The adult skeleton contains 206 bones of different sizes and shapes according to function. Bones give support to the body, protect delicate organs and act as a storage site for minerals such as calcium. In human development, the origin of bone can be dated back to the first three weeks of foetal life (see e.g. Olsen 2003). During this pre-embryonic period the cells giving rise to all structures of the body differentiate into three germ layers. These layers are the ectoderm (forming all nerve tissue and some epithelial tissue), the mesoderm (forming all connective and muscle tissue and some epithelial tissue) and the endoderm (forming some epithelial tissue).The ectoderm differentiates into the neuroectoderm and the epidermis. The neuroectoderm forms the neural tube (eventually becoming the brain and spinal cord) and the neural crest, while the epidermis covers the outside of the body. Facial bones and the cranium derive from the neural crest. The paraxial mesoderm forms the base of the skull, parietal bones and the axial elements of the ribs and vertebrae. The lateral plate of mesoderm forms the sternum and long bones.

There are two types of bone formation: intramembranous and endochondral ossification (see e.g. Olsen 2003, Lian et al. 2003). Both result in bone material with equal properties and structure. Intramembranous ossification is a direct process where osteoblasts differentiate from mesenchymal progenitors and bone is formed directly without a cartilaginous stage. This type of bone formation takes place within the skull. Endochondral ossification begins with the condensation of multipotent mesenchymal cells into structures that eventually become skeletal elements. The first chondrocytes appear at around five weeks of embryo development. In the developing cartilage, chondrocytes go through proliferation and differentiation. Eventually, proliferative chondrocytes differentiate into hypertrophic chondrocytes secreting factors such as Ihh and vascular endothelial growth factors, which are required for osteoblast differentiation (Mak et al. 2006). The hypertrophic cartilaginous region is invaded by blood vessels and osteoblastas already differentiated in the perichondrium. Osteoblasts then secrete a calcified bony matrix. The ossification of cartilaginous model begins during the third month of development. In addition to occuring during embryonic development, endochondral ossification takes place during the regeneration of bone following injury (fracture callus).

2.1.1.3 Apoptosis

The size of a cell population depends on the balance between cell production and cell loss (Kerr et al. 1972). In bone tissue, there is a fine balance between bone formation, bone resorption, bone cell proliferation and apoptosis. This balance keeps the skeletal mass nearly constant (Weinstein & Manolagas 2000). The rate of bone formation is mainly dependent on the number of osteoblasts. Osteoblast number is determined by the rate of replication from their progenitors and the lifespan of mature cells (Jilka et al. 1998). The vast majority (50-70%) of osteoblasts die of apoptosis (Jilka et al. 1999); the prevalence of osteoblast apoptosis is regarded a major regulator of bone formation (Jilka et al. 1999).

Apoptosis is a basic biological mechanism that multicellular organisms use to remove unwanted cells. The morphological phenomenon was first described by Kerr et al. (1972) to include nuclear fragmentation and chromatin condensation, plasma membrane blebbing, cell shrinkage and the eventual cell breakage into small membrane-surrounded fragments. These fragments, apoptotic bodies, are cleared by phagocytosis by other cells, without inciting an inflammatory response. In addition to removal of a cell, apoptosis gives rise to a number of signal molecules that affect the behaviour of neighbouring cells (Noble 2003). The morphological and biochemical changes associated with apoptosis are caused by activation of intracellular cysteine proteases, caspases (Reed 2000). These proteases are in the form of inactive zymogenes in virtually all animal cells. Their activation is triggered by proteolytic cleavage to generate large (~20 kD) and small (~10 kD) subunits. The active enzyme is a heterotetramer composed of two large and two small subunits, with two active sites per molecule (Thornberry & Lazebnik 1998). Caspases are activated by their own cleavage at aspartic acid, and after activation cleave their substrates at the aspartic acid residues. This leads to a proteolytic cascade whereby activated enzymes activate themselves and each other (Reed 2000). Ultimately, cell death is controlled by the balance between the apoptosis inhibitor and promoter signals (Raff 1998).

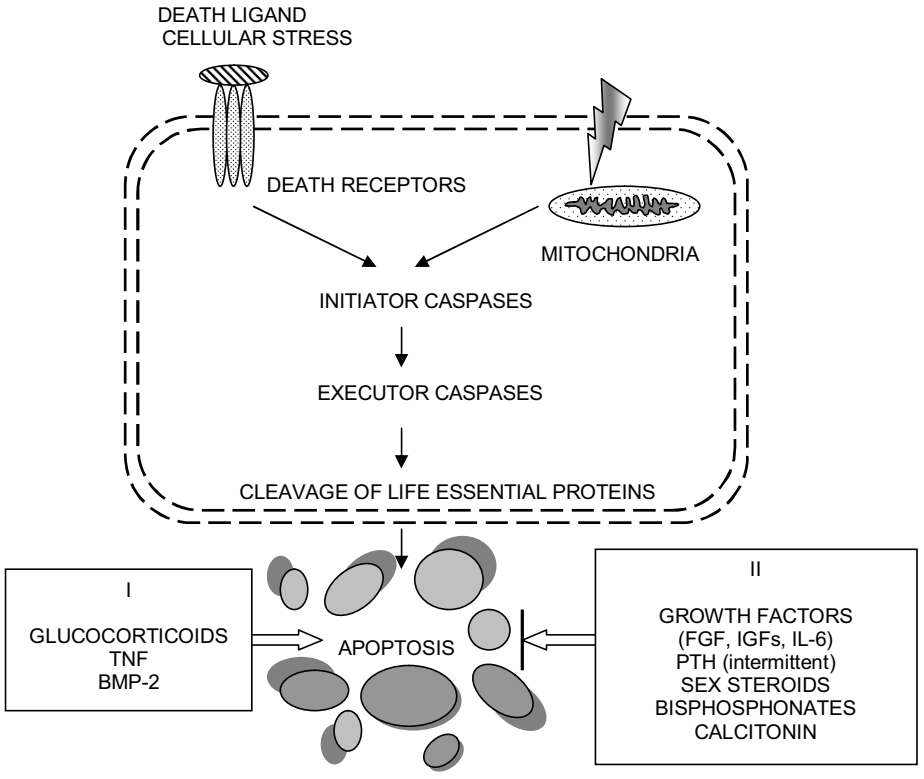

Figure 3: Apoptosis signalling (from Xing and Boyce 2005). The extrinsic signalling pathway (on the left) is triggered when a death ligand binds to the death receptor, which in turn cleaves

pro-caspase 8, resulting in an active enzyme. The intrinsic signalling pathway (on the right) is activated by the release of cytochrome c from the mitochondria due to cellular stress. Cytochrome c binds to Apaf-1, which in turn activates caspase 9. Selected factors promoting osteoblast apoptosis are presented in text box I, and factors inhibiting osteoblast apoptosis are presented in text box II.

Over a dozen caspases have been identified in humans, two-thirds of which may participate in apoptosis (Hengartner 2000). If subgrouped from a functional perspective, it is useful to view caspases as initiator (upstream) or effector (downstream) caspases. Initiator caspases are activated in response to signals ordering the cell to die. The initiators pronounce the death sentence, but the effector caspases are the true executioners that cleave the key proteins and dismantle the cell (Barinaga 1998). Effector caspase activation is largely dependent on the activation of initiator caspases.

Two complex caspase activation pathways exist the extrinsic and intrinsic pathways (Figure 3). A simplified description of extrinsic induction of apoptosis involves CD95, FADD (Fas-associated death domain) and pro-caspase 8 proteins. The signalling pathway is triggered when a CD95 ligand binds to the receptor, which in turn alters the intracellular parts of the receptor to bind adapter protein FADD. FADD binds pro-caspase 8 and cleaves it, resulting in an active enzyme.

Cells commit suicide not only when they receive signals from outside but also when a single cell fails to work properly, such as in an incomplete cell-division cycle. The intrinsic caspase activation pathway involves the release of cytochrome c from the mitochondria, thereafter cytochrome c binds to an apoptotic protease-activating factor-1 (Apaf-1) protein. Apaf-1 cleaves the initiator caspase-9 zymogen to form an active enzyme. Bone cell apoptosis can be promoted or inhibited by different stimuli. Alterations in the timing and extent of osteoblast apoptosis could have a significant impact on the rate of bone formation (Jilka et al. 1998). Furthermore, the lifespan of osteoclasts, osteoblasts and osteocytes is an important determinant of bone mass and strength (Xing & Boyce 2005).

2.1.2 Osteoclasts

Osteoclasts are large multinucleated, bone-resorbing cells needed for the removal of mineral. Osteoclast activity dictates the rate of bone resorption. Osteoclasts are formed by the fusion of mononuclear progenitor cells (Teitelbaum 2000). These precursors are of haematopoietic origin. Differentiation into mature osteoclasts requires macrophage colony-stimulating factor (M-CSF) and receptors for activation of nuclear factor kappa B (NF-κB) ligand (RANKL) (Teitelbaum 2000). These two osteoclast differentiation factors are essential for osteoclastogenesis. Both are produced by osteoblasts and marrow stromal cells. M-CSF is a secreted product that binds to its receptor, c-Fms, and allows early osteoclast precursors to continue to mature. RANKL is a surface protein, which binds to RANK in cell-to-cell contact. Binding of RANKL to RANK leads to the differentiation and maturation of osteoclast

precursor cells to mature osteoclasts. This binding results in a cascade of events that includes activation of NF-κB. Osteoclast formation and activity can be blocked by osteoprotegerin (OPG) (Lacey et al. 1998). OPG is a soluble decoy receptor competing with RANK for RANKL. When OPG binds to RANKL, it prevents it from binding to RANK, resulting in the inhibition of osteoclast formation. Almost all of the bone-resorbing factors stimulate expression of RANKL in osteoblasts/stromal cells (Suda et al. 1999). Three independent signals have been proposed to induce RANKL expression in osteoblasts/stromal cells: vitamin D receptor-mediated signals induced by $1,25(OH)_2D_3$, cAMP/protein kinase A (PKA)-mediated signals induced by PTH or PGE_2, and gp130-mediated signals induced by IL-11 (Suda et al. 1999). Among these signals, vitamin D receptor- and cAMP/PKA-mediated signals suppress OPG expression in osteoblasts/stromal cells.

2.1.3 Bone remodelling

Once bone formation (i.e. the acquisition of bone mineral density, BMD) has stopped (somewhere about 28 years of age), peak bone mass (PBM) is maintained by remodelling. Remodelling is a continuous process involving the breakdown and re-formation of bone, the maintanance of maximal BMD and the repair of any damage (e.g. fractures). The adult skeleton is completely regenerated every 10 years (Manolagas 2000).

Bone remodelling takes place in basic multicellular units (BMUs). The BMU is a unique temporary anatomical structure that consists of a team of osteoclasts in front; a team of osteoblasts behind and the associated blood vessels, connective tissue and nerves (Parfitt 1994). Each BMU originates in a particular place at a particular time, travels through or across the bone surface towards its target and continues beyond this target for a variable distance (Parfitt et al. 1996). The advance of a BMU continues for 6-9 months (Jilka 2003).

A BMU will originate after microdamage to the bone, mechanical stress, exposure to some cytokines or at random. The initiation signals producing a BMU are not fully understood, but osteocyte involvement has been suggested (Noble et al. 1997). The lining cells first become active (in response to osteocyte signals) and change from a pancake-like shape to a cuboidal form. Lining cells may then prepare the bone surface for osteoclast attachment (Manolagas 2000). Upon contact with bone, osteoclasts develop a polarized phenotype and attach firmly to the surface (Fallon et al. 1983). The bone-facing plasma membrane forms a ruffled border, sealed from the sides with a sealing zone. The resorptive process takes place in this isolated, extracellular microenvironment at the osteoclast-bone interface. Osteoclasts produce acid to promote mineral mobilization and to establish the pH optimum for lysosomal enzymes that degrade the protein matrix (Blair et al. 1986, Blair et al. 1993). The most abundant protease enzyme in osteoclasts is cathepsin K (Drake et al. 1996). Cathepsin K is regarded as a key enzyme in the degradation of organic bone matrix (Morko et al. 2005). Excessive cathepsin K enhances bone resorption and leads to osteopenia of trabecular bone and increased porosity of cortical bone (Morko et al. 2005, Kiviranta et al. 2001). The average lifespan of an osteoclast

is about 12 days, after which it undergoes a programmed cell death, known as apoptosis (Jilka 2003).

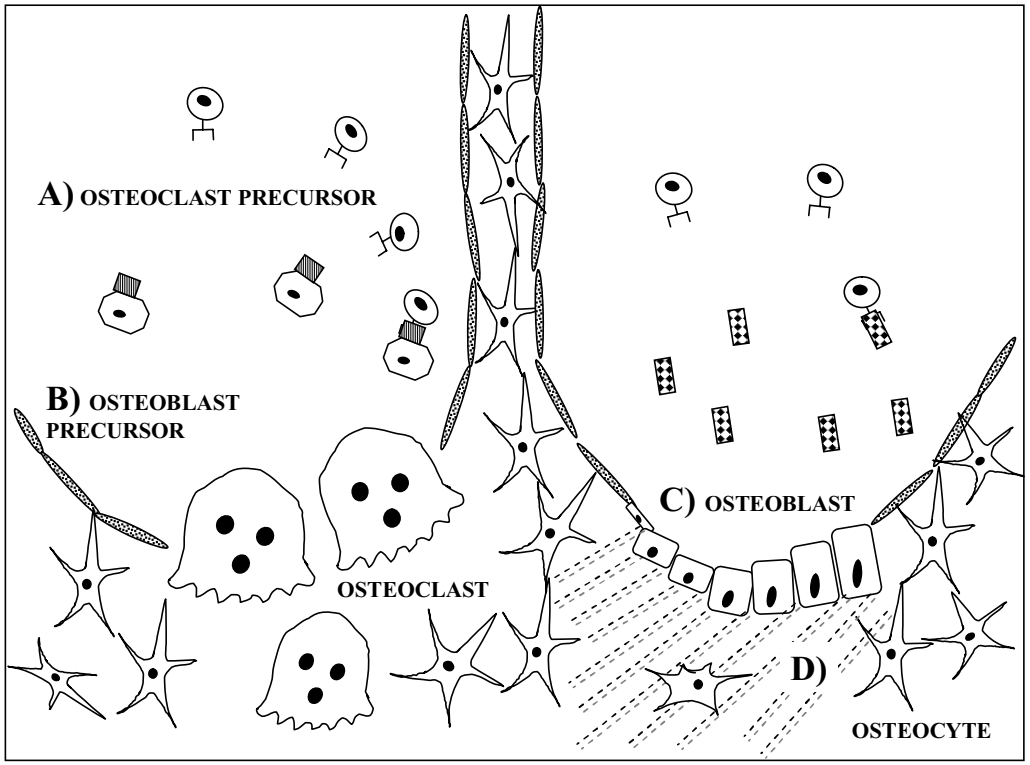

Figure 4: Bone remodelling. A) Osteoclast precursor cells have a receptor for RANKL. Osteoblast precursor cells have a ligand for RANK. B) Upon binding RANKL, osteoclast precursors differentiate into mature bone-resorbing osteoclasts. C) Mature bone forming osteoblasts secrete RANKL decoy receptor OPG, which inhibits osteoclast differentiation and function. D) Some of the osteoblasts become entrapped in the newly synthesized matrices and further differentiate into osteocytes.

As the BMU wanders, new osteoclasts are continuously activated and then start resorption. At any site on the surface, the resorption lasts about two weeks. The coordination of osteoblast and osteoclast differentiation, cell function and cell death is essential for the maintenance of normal bone homeostasis. IGF-I is a candidate for the coupling factor in bone resorption and formation (Rubin et al. 2002). The development of mature osteoblasts at the BMU is promoted by growth factors released from the bone matrix during resorption, as well as by growth factors produced by osteoblast progenitors themselves. The active, secreting osteoblasts make layers of osteoid, slowly refilling the cavity, and, as the osteoid thickens it begins to mineralize (Figure 4). Mineralization, also, is regulated by the osteoblasts. The osteoblasts are less efficient at making bone than the osteoclasts are at removing it, and hence, any factor causing a higher rate of bone remodelling will ultimately lead to a more rapid loss

of bone mass and more fragile bones. Bone loss caused by sex steroid deficiency or glucocorticoid excess is caused by alteration of bone cell production by prolonging osteoclast lifespan and shortening osteoblast lifespan. The average lifespan of an individual matrix-synthesizing osteoblast varies from a few days to about 100 days (Jilka 2003). During bone remodelling the osteoblast lifespan is estimated to be 6-8 days (Jilka et al. 1998). The final osteoblasts turn into lining cells, osteocytes or die of apoptosis.

2.2 Bone strength and quality

Weak bones fracture easily. Osteoporosis is a disease characterized by low BMD and increased fracture risk. Bone strength is a combination of structural, geometric and material properties (van der Meulen et al. 2001). Bone size, shape and particulalarly mass are important determinants of bone strength (Heaney et al. 2000). Big bones have higher absolute strength than small bones, but their relative strength can be identical if the material properties are equal. The most important material property of bone is the mineral content; the more mineral in the bone, the greater the stiffness and ultimate strength (Seeman 2003). Bone toughness is determined by collagen content (Currey 2003); decreased collagen in bone reduces toughness (Seeman 2003).

Bone mineral content can be measured by dual-energy x-ray absorptiometry (DXA). The measurement provides two-dimensional areal BMD. DXA measurement is widely used in clinical assessment of BMD and individual fracture risk. Peripheral quantitative computed tomography (pQCT) gives three-dimensional volumetric BMD. pQCT discriminates between cortical and trabecular bone, and the results provide more information for research purposes than DXA. Microtomography (micro-CT) also gives three-dimensional information on bone. Micro-CT scans bone trabeculae and even bone resorption sites.

Bone mechanical strength is probably the most important parameter related to fracture risk (Peng et al. 1994). Animal models are used for the mechanical testing of bone, which requires applying force until bone breaks. The tibia bending test provides information on cortical bone material properties, and the mechanical test for the femoral neck is used to measure cancellous bone properties (Ruhmann et al. 2006). In cancellous bone, the overall structure seems to be more important than the material properties (Currey 2003).

Bone quality generally refers to the effects of skeletal factors that contribute to bone strength but are not accounted for by measures of bone mass (Hernandez & Keaveny 2006). The amount of bone turnover influences bone strength. A sufficient amount of turnover is needed to replace old bone with new bone. However, the remodelling process itself is a source of structural weakness (Heaney 2003). With high remodelling, older bone accumulates microdamage (Schaffer 2003) and younger bone becomes undermineralized. Microdamage accumulation reduces bone stiffness (Noble 2003). Excessive remodelling will eventually lead to bone loss, trabecular thinning, loss of connectivity, cortical thinning and porosity (Bourrin et al. 2000, Seeman 2003). In the regions of high tissue strain, resorption cavities

reduce the strength and stiffness of the bone independent of their effect on bone volume (Hernandez et al. 2006).

2.3 DIET

2.3.1 Diet and bone

Nutrition is important for optimizing bone health throughout life. The dietary components most widely studied and regarded as relevant to bone health are calcium and vitamin D. Several other nutrients also play a role in bone health, including minerals, such as phosphorus and magnesium, micronutrients, such as vitamin K and vitamin C, and macronutrients, such as protein. Because bone is a complex living tissue, examining nutrients individually may yield misleading. Nutrients are found together in foods, and associations attributed to a single nutrient may, in fact, be caused by a more complex constellation of nutrients consumed contemporaneously (Tucker et al. 2002). In addition to sufficient intake of specific nutrients, food groups (e.g. fruit and vegetables) seem to have a positive effect on bone health (New et al. 2000, Tucker et al. 2002, MacDonald et al. 2004, Okubo et al. 2006). Eating pattern can affect the intake of nutrients that build bones. For example, calcium is absorbed by an active saturable system that is affected by the source and amount of calcium. Calcium bioavailability from plant foods is reduced if the plant contains a calcium absorption inhibitor such as oxalate or phytate (Weaver et al. 1999).

Eating frequency also plays another role besides the absorption of nutrients. In a rodent model, frequent small meals increased total bone mineral content, trabecular bone mineral density and cortical thickness, and markedly reduced the decrease in these parameters in aged rats (Li & Muhlbauer 1999). Gastrointestinal hormones released after a meal are also involved in regulating bone turnover (for review see, Clowes et al. 2005). Hormones, such as glucagon-like peptide (GLP)-1 and -2, have been reported to cause immediate reduction in bone resorption after a meal (Henriksen et al. 2003). Gastric inhibitory polypeptide (GIP), another gastrointestinal hormone, has receptors in osteoblasts (Bollag et al. 2000). GIP increases bone formation by increasing collagen I synthesis and ALP activity in osteoblasts (Bollag et al. 2001) and by reducing osteoblast apoptosis (Tsukiyama et al. 2006). Figure 5 summarizes the dietary factors affecting bone health.

The positive effects of foods or nutrients may be addressed through a nutritional pathway or modulation of other factors affecting bone health. Examples of such nutritional effects include the hormonal functions of phytoestrogens, reduced risk of falls due to the individual's increased alertness in response to the substance (e.g. caffein), and the influence of food components, such as bioactive peptides, on gene expression. A Western-style diet that is low in calcium, vitamin D and folic acid and high in fat may result in low bone mass and poor bone quality, leading to an increased risk of a fragility fracture. Ward et al. (2003) have

shown that Western-style diets have a negative effect on femur BMC and biomechanical strength properties in a rodent model.

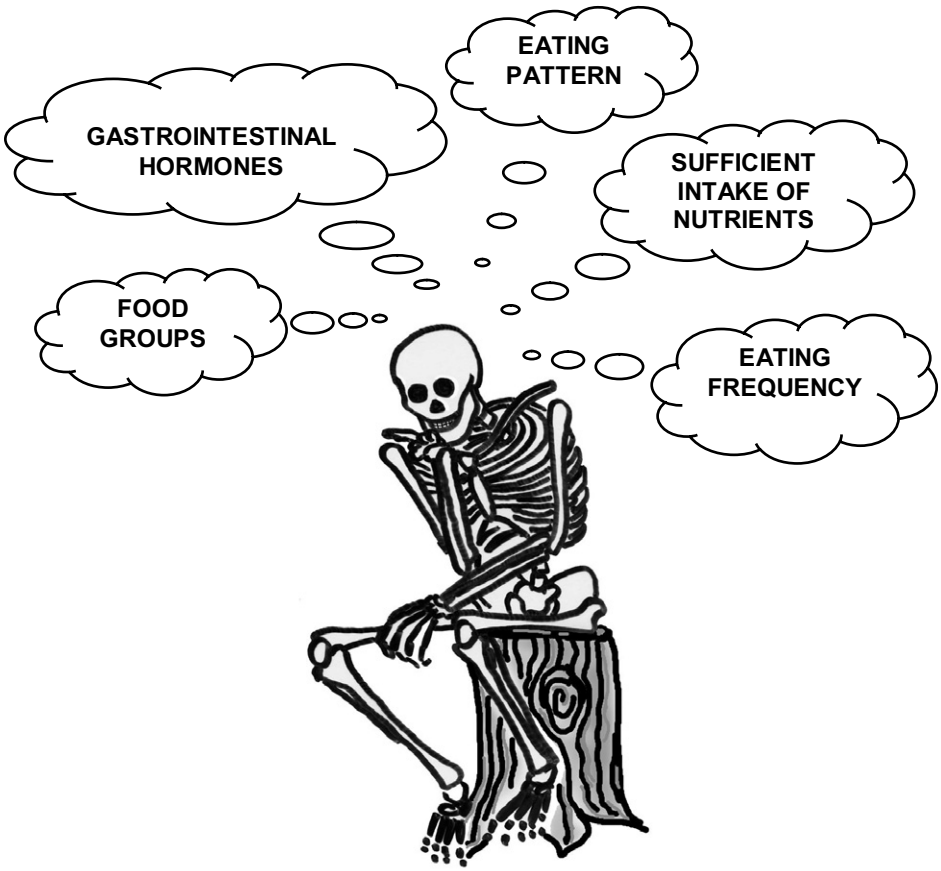

Figure 5: Dietary factors affecting bone health.

2.3.2 Nutrients and gene expression

Individual variation in gene expression is a combination of genetic polymorphisms, physiological variations (such as time of day and gender) and environmental factors (Hamilton 2002). Diet is an environmental factor affecting gene expression through epigenetic modification (Jaenish & Bird 2003). Epigenetic information is heritable during cell division, but it is not contained within the DNA sequence itself, hence the name meaning 'outside conventional genetics'. Epigenetic processes are mediated by DNA methylation and histone modifications (Jaenish & Bird 2003). Studies with monozygotic twins help to estimate the size of the contribution of genetic and environmental factors to the natural variation in gene expression. Phenotypic differences within monozygotic twin pairs are due to environmental effects alone, as these individuals uniquely share their entire genetic

background (MacGregor et al. 2000). Fraga et al. (2005) examined gene expression among 3-to-74-year-old twin pairs using microarray technology. They found that although 3-year-olds had nearly identical expression profiles, the 50-year-old twins had very different profiles. The key message of this study was that epigenetic modifications in monozygotic twin pairs diverge as they become older, with this difference most likely being explained by both internal and external factors (Fraga et al. 2005). Environmental factors, such as diet, can modify the epigenetic states and contribute to the formation of the phenotype (Jaenish & Bird 2003).

Nutrients regulate gene expression by direct and indirect mechanisms. Direct interaction with transcription factors controlling specific genes involves cis-regulatory elements in the promoter region. Most nutrients function by regulating the secretion or intracellular action of hormones, which in turn alter gene expression (De Caterina & Madonna 2004). Macro- and micronutrients regulate the overall cell metabolic functions by controlling their own metabolism (e.g. glucose - insulin) and changing the phenotypic response to the environment (e.g. vitamin A - variety of genes). Biological processes can be finely tuned by nutritional interventions in order to restore appropriate biological responses. This kind of nutrigenomics is a scientific investigation of the effects of nutrients in the body on each of us as individuals. The future of nutrition might involve diets tailored to suit a person's genetic make-up, including nutraceuticals (also referred to as phytochemicals or functional foods) with health-promoting, disease-preventing or medicinal properties.

A balanced diet, adequate energy and nutrient intake form the foundation for all tissue development, including bone. Optimal nutrition enables maximal PBM development and maintains the bone mass achieved. As bone can be influenced by diet, nutrients important for bone growth should be recommended, but greater awareness regarding bone-impairing nutrients is also needed (Ward et al. 2003). Dietary phosphate, protein, and bioactive peptides will be discussed in more detail below.

2.3.3 Phosphate and bone cells

Phosphorus is the second most abundant element (after calcium) present in our bodies; 85% is found in the bones and teeth. Calcium and phosphorus work together in bone formation, with the Ca:P ratio in human bone being approximately 1.5:1 (w:w) (European Food Safety Authority 2005) hence inorganic phosphate (Pi) is a necessary component of hydroxyapatite. Pi functions also as a signalling molecule (Beck 2003). Osteoclasts and osteoblasts both have Na-Pi transporters.

Pi is integral to bone remodelling and osteoblast function during the differentiation process (for review, see Beck 2003). Increased intracellular Pi upregulates osteoblast genes involved in cell cycle, proliferation and energy metabolism (Conrads et al. 2005). As osteoblast differentiation proceeds, the levels of ALP enzyme rise. ALP is responsible for the locally increased Pi levels and the initiation of mineralization. Osteoblast Na-Pi transporter activity

can be upregulated by PTH. An experiment on UMR-106 cells showed 10^{-7} M PTH to stimulate the Na-Pi transport (Seltz et al. 1989). Similarly, intermittent administration of PTH stimulates osteoblast proliferation and differentiation (Dempster et al. 1993, Swarthout et al. 2001).

Phosphate participates in the remodelling process. At bone resorption site, osteoclasts dissolve the bone matrix and release calcium- and phosphate-ions. A relatively small increase in the amount of calcium (0.1-1 mM) together with Pi causes osteoblast apoptosis (Adams & Shapiro 2003). Osteoblast apoptosis occurs most frequently at the sites of active bone resorption (Adams & Shapiro 2003). As bone resorption proceeds, increasing concentrations of Pi suppress osteoclasts (Kanatani et al. 2003). In vitro high Pi concentration (2.5-4 mM) inhibits osteoclast formation and bone resorption by mature osteoclasts (Kanatani et al. 2003). Pi inhibits osteoclast maturation by direct action and via osteoblasts by the upregulation of OPG (Kanatani et al. 2003). The increase in osteoblast differentiation and decrease in osteoclast formation and activity by Pi promotes local bone formation stimultaneously blocking bone loss.

In vivo Katsumata et al. (2005) have shown a high phosphate diet to stimulate osteoblast RANKL mRNA expression through elevated PTH concentration. The increase in RANKL/OPG ratio enhances osteoclastogenisis and results in an increased osteoclast number, causing bone loss via increased bone turnover.

2.3.4 Dietary phosphate and bone

Phosphorus functions as a limiting nutrient in the biosphere. A common feature of such nutrients is efficient absorption; phosphate absorption is 60-80% remaining unchanged even at high intakes (Draper et al. 1972, for review, see Lemann & Favus 2003). Dietary phosphorus is predominantly found as phosphate (PO_4^{3-}). Phosphate absorption occurs in the small intestine via a transcellular pathway that is dependent on sodium (Na- Pi cotransport) (reviewed in Cross et al. 1990). A low-phosphate diet and vitamin D_3 increase brush-border Na- Pi cotransporter in the small intestine (Hattenhauer et al. 1999). Phosphate homeostasis and plasma concentration are maintained by the kidneys. Most of the phosphate is excreted into the urine. Phosphate reabsorption takes place in the proximal tubule and is mediated by Na-Pi co-transporter type IIa (Npt2a) (for review, see Tenenhouse 2005). Phosphate reabsorption is increased by factors such as a low dietary phosphate intake, insulin-like growth factor (IGF)-I and 1,25-dihydroxyvitamin D_3, whereas PTH and fibroblast growth factor (FGF)-23 inhibit phosphate reabsorption, resulting in phosphaturia.

In human nutrition, phosphate is widely distributed in many foods (Heaney 2004), and is thus no longer a limiting nutrient. The main sources of intake for phosphate in the Finnish diet are milk and milk products (35%), bread and cereal (30%) and meat and eggs (18%) (National FINDIET 2002 Study). Convenience foods and soft drinks also contribute to the phosphate

intake in the form of phosphorus-containing food additives. Already in the late 1970s food additives accounted for 20-30% of adult phosphorus intake (Calvo 1993). Because the nutrient composition databases used in calculating mineral intakes do not take into account phosphorus-containing food additives, intakes are likely to be underestimated (Calvo 1993). The recommended dietary allowance (RDA) for phosphate in Finland (600 mg/d for adults, Finnish Nutrition Recommendations 1999) is exceeded on average by two- to threefold (Findiet 2002). The average dietary calcium to phosphate ratio in Finnish diet is 1:2 for women and 1:3 for men. Because phosphorus is abundantly distributed in the food supply and is well absorbed, dietary phosphorus deficiency is extremely rare (Institute of Medicine 1997). Dietary phosphate deprivation leads to rapid breakdown of the skeleton (e.g. Kanatani et al. 2003). At the cellular level, bone formation processes are inhibited and bone resorption is stimulated. In vivo high dietary phosphate intake has been shown to cause bone loss in a variety of animal models (Calvo & Park 1996). Studies in rodents (mice and rats), rabbits, pigs, dogs and primates show high dietary phosphate diet to elevate serum PTH, stimulate bone resorption, and to cause bone loss and osteopenia (Calvo & Park 1996).

2.3.5 Dietary protein and bone

Protein accounts for one-third of bone mass, making it one of the most protein-dense tissues of the body. Nevertheless, high dietary protein intake has been claimed to have detrimental effects on bone health (for review, see Ginty 2003). A proposed underlying mechanism is that increased protein intake increases acid production due to the hepatic oxidation of sulphur-containing amino acids such as methionine, cysteine and cystine (Rizzoli & Bonjour 2004). This would be the case in renal diseases, where a high-protein diet will cause metabolic acidosis as the renal response to excrete excess acid is reduced. The consequential reduction in blood pH increases bone resorption and urinary calcium loss (Krieger et al. 2004). Bone resorption is increased since reduction in pH increases osteoclast formation and activity and reduces osteoblast collagen synthesis and mineralization (Arnett et al. 1994, Lehenkari et al. 1998, Arnett 2003).

The in vivo studies showing high dietary intake to increase bone resorption in healthy subjects are often carried out using high doses of purified forms of protein. In mixed diets the dietary alkali load (fruit and vegetables) has been shown to neutralize the pH-lowering effects (for review, see Ginty 2003). Furthermore, there is no definitive evidence that high protein intake increases renal calcium excretion in healthy subjects. In fact, dietary protein increases intestinal calcium absorption (Kerstetter et al. 2005), whereas low protein intake reduces calcium absorption and results in elevated serum PTH (for review, see Ginty 2003). The recommended amount of protein for healthy adults is 15-20 energy% or 0,8–1 g/kg body weight (Finnish Nutrition Recommendations 2005).

Many studies have found an overall beneficial relationship between high dietary protein and bone health (Hannan et al. 2000, Hoppe et al. 2000, Whiting et al. 2002, Kerstetter et al. 2003, Bowen et al. 2004, Wengreen et al. 2004, Alexy et al. 2005). Dietary protein has a positive

correlation with bone area (Hoppe et al. 2000) and BMD, and a negative correlation with fracture rate (Hannan et al. 2000, Dawson-Hughes & Harris 2002). Dietary protein plays an important role in bone strength (Oxlund et al. 1995, Wengreen et al. 2004), as it is essential for bone turnover and matrix formation (Oxlund et al. 1995, Heaney 2002). Furthermore, protein supplementation has been proven to aid fracture repair in hip fracture patients (Delmi et al. 1990).

The osteotrophic effects of protein are partly explained by enhanced IGF-I production and action (Thissen et al. 1994, Bonjour 2005). IGF-I is an essential mediator of tissue anabolism (for review, see Ginty 2003). It increases osteoblast activity and type I collagen production and acts as a coupling factor for bone resorption and formation (Rubin et al. 2002). Furthermore, IGF-I is essential for longitudinal bone growth, as it stimulates the proliferation and differentiation of chondrocytes in the epiphyseal plate. However, in a rodent model, a protein-restricted diet with normalized IGF-I by infusion failed to promote growth, indicating adequate protein intake to have positive effects in addition to increased IGF-I (Thissen et al. 1994). Adequate protein intake should be recommended in the maintenance of bone integrity and in the prevention and treatment of osteoporosis (Bonjour 2005).

2.3.6 Bioactive peptides and bone

Peptides are short chains of amino acids produced in the hydrolysis of proteins by gastrointestinal enzymes or by microbiological fermentation. A small proportion of these peptides are resistant to further degradation and possess biological function beyond being tissue-building blocks. These peptides are called bioactive peptides. Some of these peptides express activity in the intestine (Shimizu 2004), others after absorption (Satake et al. 2002). Two human di-/tri-peptide transporters have been identified, hPepT1 and hPepT2 (for review, see Nielsen & Brodin 2003). hPepT1 is expressed in the small intestine, whereas both hPepT1 and hPepT2 are expressed in the proximal tubule.

Bioactive peptide actions vary; opioid peptides are opioid receptor ligands and modulate absorption processes in the intestinal tract, angiotensin-I-converting enzyme (ACE)-inhibitory peptides function as hemodynamic regulators and exert an antihypertensive effect, casein peptides are immunomodulators and stimulate the activities of cells of the immune system, antimicrobial peptides kill sensitive microorganisms, antithrombotic peptides inhibit aggregation of platelets, and caseinophosphopeptides may function as carriers for different minerals, especially calcium (Froetschel et al. 2001, Aimutis 2004, FizGerald et al. 2004). Peptides remaining functional after absorption are typically 2-5 amino acids in length and have a proline residue in the C-terminus (Curtis et al. 2002). This structure is common among food-derived tripeptides such as Ile-Pro-Pro (IPP), Val-Pro-Pro (VPP) and Leu-Lys-Pro (LKP). Food-derived bioactive peptides are claimed to be health-enhancing components of functional food and pharmaceutical preparations (Meisel & Bockelmann 1999). Estimates of peptide consumption are difficult to obtain because of the wide variation in concentrations (Bernard 2005). However, in Japan it is estimated that the mean intake from tripeptide-

enhanced products is 0.005 mg/kg body weight/day and in United States 0.1 mg/kg body weight/day (Bernard 2005).

Another application for the use of bioactive peptides is the development of biomaterials for tissue engineering. The surface modification of biomaterials with bioactive molecules is a simple way to make biomimetic materials, i.e. materials elicting specific cellular responses and directing new tissue formation (Lutolf et al. 2003). The early biomaterial work has been done on long ECM proteins, such as fibronectin and lamin, which promote cell adhesion and proliferation. Since the several amino acids long signalling domains were found along the long chain of ECM proteins, these fragments have been used for surface modification in numerous studies (for review, see LeBaron & Athanasiou 2000). These amino acids interact with cell membrane receptors. The short peptide sequences are more stable and can be synthesized more economically than long proteins. Materials such as glass, quartz, metal oxide and polymers have been modified with peptides and characterized for cellular interaction with their surfaces. The most commonly used peptide for surface modification is the Arg-Gly-Asp (RGD) amino acid sequence derived from fibronectin. RGD serves as a primary cell attachment cue (for review, see Schaffner & Dard 2003). Osteoblasts have integrin receptors which bind RGD (reviewed in Schaffner & Dard 2003). This binding site is involved in cell morphology, differentiation, proliferation, gene expression, cell survival and apoptosis. The RDG application in tissue engineering uses the same pathways that take place in bone remodelling in vivo. As osteoclasts resorb bone, minerals are dissolved and matrix proteins are degraded into amino acid sequences. Some of these sequences, such as RGDS and GRGDSP (Adams & Shapiro 2003), cause osteoblast apoptosis and clear the way for osteoclasts and some of the peptides, such as RDG, to promote osteoblast cell attachment, proliferation, differentiation and mineralized matrix formation. Furthermore, RGD peptide inhibits bone resorption; hence, local bone formation can efficiently repair the resorption pit (for review, see Schaffner & Dard 2003). The fact that peptides containing the RGD motif arrest osteoclastic bone resorption in vitro (Horton et al. 1991) suggests such agents may also blunt bone resorption in vivo (Engelman et al. 1997).

Integrin receptors are not the only mechanism of action for peptides to modulate osteoblast function. The extracellular calcium-sensing receptor (CaR) plays an important role in the regulation of calcium homeostasis. CaR is widely expressed in many tissues, including bone. Several osteoblastic cell lines have been found to express CaR (Yamauchi et al. 2005), but there is still debate whether all osteoblasts possess CaR (Pi et al. 2005). Although calcium is the physiological ligand for CaR, other divalent and trivalent cations can also activate this receptor (Pi et al. 2005). In addition, CaR activity (in the presence of Ca^{2+}) is modulated by amino acids (Conigrave et al. 2000) and small peptides (Quinn et al. 1997). The CaR regulates bone turnover by stimulating osteoblast proliferation (Yamaguchi et al. 1998) and differentiation (Yamauchi et al. 2005). The CaR also modulates chondrogenic cell matrix production and mineralization (Chang et al. 2002). Because the CaR can integrate signals arising from distinct classes of nutrients i.e. mineral ions and amino acids, the actions of

amino acids on the CaR may provide explanations for several long-recognized but poorly understood effects of dietary protein on calcium metabolism (Conigrave et al. 2000). The CaR might also be a mechanism of action for bioactive peptides.

2.4 PARATHYROID HORMONE (PTH)

2.4.1 PTH metabolism

Parathyroid hormone (PTH) is a single-chain polypeptide with 84 amino acids. The hormone is generated by the chief cells of the parathyroid glands and released into circulation. PTH(1-84) is further cleaved to generate shorter fragments. The N-terminal 1-34 fragment of the PTH mediates full PTH activity.

PTH functions as the principal regulator of calcium homeostasis (Figure 6). Parathyroid cells have calcium-sensing receptors (CaRs). The CaR is negatively coupled to PTH release (for review, see Kifor et al. 2002). The parathyroid cell is sensitive to changes in extracellular calcium concentrations, with small increases in extracellular calcium inhibiting secretion of PTH. Conversely, a decrease in extracellular calcium leads to a rapid increase in PTH secretion.

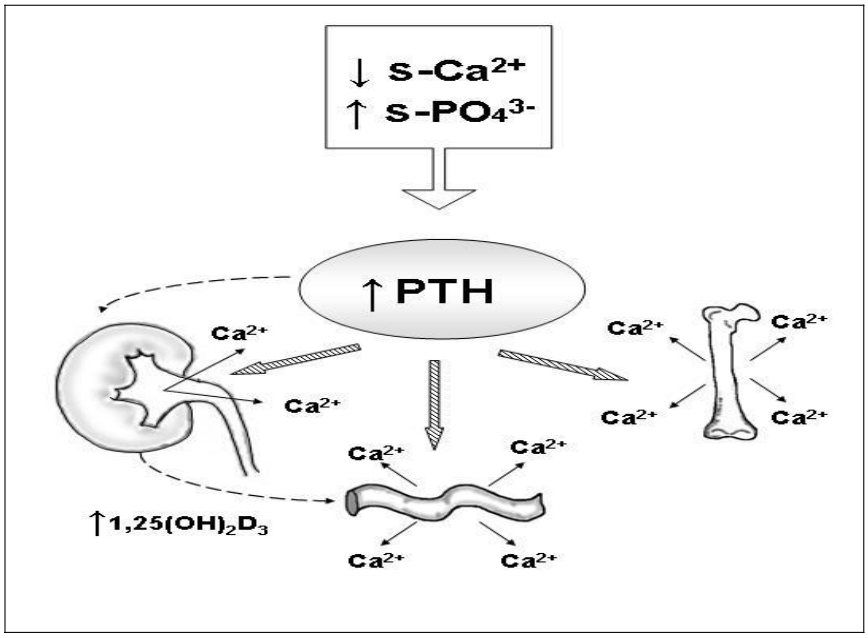

Figure 6: PTH regulates extracellular calcium. Parathyroid cells respond to decreased serum calcium and/or increased serum phosphate by increaseing PTH secretion. PTH increases calcium reabsorption from the renal tubules, stimulates renal $1,25(OH)_2D_3$ production and

intestinal calcium absorption. PTH stimulates bone resorption by increasing the number and activity of osteoclasts. As a result serum calcium is returned to normal.

Phosphate also has a direct action on the parathyroid gland, although the mechanism remains somewhat obscure. High phosphate concentration inhibits arachidonic acid production in the parathyroid tissue, which in turn stimulates PTH secretion (Almadén et al. 2000). A high-phosphate diet induces parathyroid cell proliferation (Roussanne et al. 2001) and PTH secretion, causing serum PTH levels to rise (Almadén et al. 1996, 1998, Kärkkäinen & Lamberg-Allardt 1996, Miyamoto et al. 1998, Koshihara et al. 2001).

An increase in phosphate intake can lower serum ionized calcium, which further enhances PTH secretion (for review, see Lemann & Favus 2003). Reducing dietary phosphorus intake reduces PTH (Rodriguez 1999). High dietary phosphate intakes might also form insoluble salts in the intestinal lumen and reduce calcium absorption (e.g. Masuyama 2003). However, according to the Institute of Medicine (1997), phosphorus does not appear to negatively affect calcium absorption. Moreover, while the intake ratio of calcium to phosphorus is relevant during infancy and periods of rapid growth, it is not important in adulthood (Institute of Medicine 1997). The principal target organs for PTH are the kidney (increasing proximal tubular resorption of calcium, phosphate excretion and 1,25 dihydroxyvitamin D formation) and the skeleton (for review, see Swarthout et al. 2002).

2.4.2 PTH and bone metabolism

Osteoblasts are the primary target cells for the effects of PTH on bone tissue. For long it was believed that osteoblasts express the PTH1 receptor, while osteoclasts do not (Partridge et al. 1981, Suda et al. 1996). However, recent findings indicate that osteoclasts also have PTH1 receptors (Dempster et al. 2005). For a review on PTH1 receptor structure and function, see Chorev (2002). PTH effects on osteoblasts are mediated via binding of PTH to the seven membrane-spanning G protein–coupled receptor and activation of either the protein kinase A (PKA) pathway or the protein kinase C (PKC) pathway (Kronenberg et al. 1998).

The PKA pathway is mediated via Gs subunit and it involves adenylate cyclase activation, followed by cAMP formation, and PKA activation (Turner et al. 2000). This pathway is activated in PTH's anti-apoptotic function but also in RANKL production (Bellido et al. 2003). The PKC pathway is mediated via Gq subunit (Turner et al. 2000). Gq activates phospholipase C (PLC), leading to the formation of diacylglycerol (DAG) which activates protein kinase C (PKC) and 1,4,5-inositol triphosphate (IP3), resulting in increased intacellular Ca^{2+} . The proliferative response of osteoblasts to PTH is mediated by the PKC pathway (Swarthout et al. 2001).

PTH-related peptide (PTHrP) is another molecule known to act on the same PTH1 receptor as PTH (Juppner et al. 1991, Lanske et al. 1998) and capable of increasing bone mass when administered intermittently (Hock et al. 1989, Caulfield et al.1990). PTH can be regarded as

the hormone to regulate calcium homeostasis by promoting bone resorption, whereas PTHrP functions as a bone cytokine to control bone mass (Martin 2005). Mesenchymal cells differentiating into osteoblasts produce PTHrP at an early stage, with decreasing levels as cells mature. PTH1 receptor is expressed at a later stage of pre-osteoblast differentiation (Martin 2005). PTHrP acts on PTH1 receptor-positive pre-osteoblasts and the cells further differentiate into mature osteoblasts. Osteoblasts can synthesize PTHrP in response to PTH (Walsh et al.1997). This locally produced PTHrP is important for the orderly commitment of precursor cells towards the osteogenic lineage and for their maturation and function (Miao et al. 2005). Furthermore, PTHrP prevents osteoblast and osteocyte apoptosis and promotes osteoclast formation (Miao et al. 2005). Within the skeletal microenvironment, PTHrP regulates bone turnover by potentiating bone formation (Miao et al. 2005). Prolonged stimulation of preosteoblasts by PTHrP increases RANKL production and osteoclast formation (Martin 2005). In vitro study by Kaji et al. (1995) revealed that the C-terminal PTHrP peptide fragments stimulate osteoclast formation in bone cell cultures containing osteoblasts, but not the bone resorption by mature osteoclasts. Previous studies by Fenton et al. (1991, 1994) reported the same PTHrP fragment (107-111) to inhibit osteoclast formation and resorption. The discrepancy could be due to different culture conditions and species.

PTH can promote both the formation and resorption of bone. Elevated levels of PTH increase bone turnover, leading to either anabolic or catabolic effects on the skeleton depending upon the pattern and duration of elevation (for review, see Poole & Reeve 2005).

2.4.2.1 Anabolic effects of PTH on bone

Intermittent administration of human PTH increases bone mass (Black et al. 2003, Arita et al. 2004). Anabolic effects require brief exposures to higher-than-average PTH concentrations. Intermittent stimulation by PTH promotes bone formation on the periosteal, endocortical and trabecular surfaces, increasing cortical and trabecular thickness and trabecular connectivity (Jiang et al. 2002). PTH also affects bone microarchitecture by shifting trabecular structure to a more plate-like appearance (Jiang et al. 2002).

PTH increases bone formation by stimulating the proliferation and differentiation of osteoblast precursors (Dempster et al. 1993, Swarthout et al. 2001), promoting osteoblast protein synthesis (Swarthout et al. 2001) and increasing osteoblast lifespan by preventing osteoblast apoptosis (Jilka et al. 1999). Intermittent administration of PTH stimulates bone formation by preventing osteoblast apoptosis, hence osteoblast matrix synthesis is prolonged (Jilka et al. 1999). The anti-apoptotic effect requires the hormone to bind to the PTH/PTHrP receptor. Osteoblasts uniformly produce cAMP in response to PTH, though it is likely that the anti-apoptotic signal is mediated by cAMP (Jilka et al. 1999). Contrary to several other studies (Calvi et al. 2001, Xing & Boyce 2005), Jilka et al. (1999) did not confirm PTH to increase bone formation through stimulating osteoblast progenitor cell proliferation or bone lining-cell hypertrophy and to resume matrix synthesis.

2.4.2.2 Catabolic effects of PTH on bone

Continuously high levels of PTH reduce BMD. Such continuous secretion of PTH (as that occuring in primary hyperparathyroidism) can lead to bone destruction. In vitro studies on cell cultures indicate the deleterious effects of PTH on bone to be mediated by increases in the number and activation of osteoclasts (Lee & Lorenzo 1999, Khosla 2001, Huang et al. 2004) and increased osteoblast apoptosis (Fujimori et al. 1992, Turner et al. 2000). In vivo continuous PTH administration does not appear to increase osteoblast apoptosis (Bellido et al. 2003). Generally, continuous PTH treatment of osteoblasts results in decreased expression of many of the genes involved in bone formation (type I collagen, ALP, osteopontin, osteonectin), while simultaneously increasing the expression of some of the genes involved in bone resorption (collagenase-3, IL-6, IGF-1) (for review, see Swarthout et al. 2002). In addition to increased collagenase expression and decreased collagen synthesis, PTH has a direct stimulatory action on human osteoclasts mediated by PTH1R (Dempster et al. 2005) and also an indirect effect via osteoblasts (reviewed in Teitelbaum 2000). Continuous stimulation by PTH results in an increase in RANKL and M-CSF expression, production of IL-6, and a decrease in OPG expression, leading to osteoclastogenesis (Ma et al. 2001, Locklin et al. 2003) and increased bone resorption.

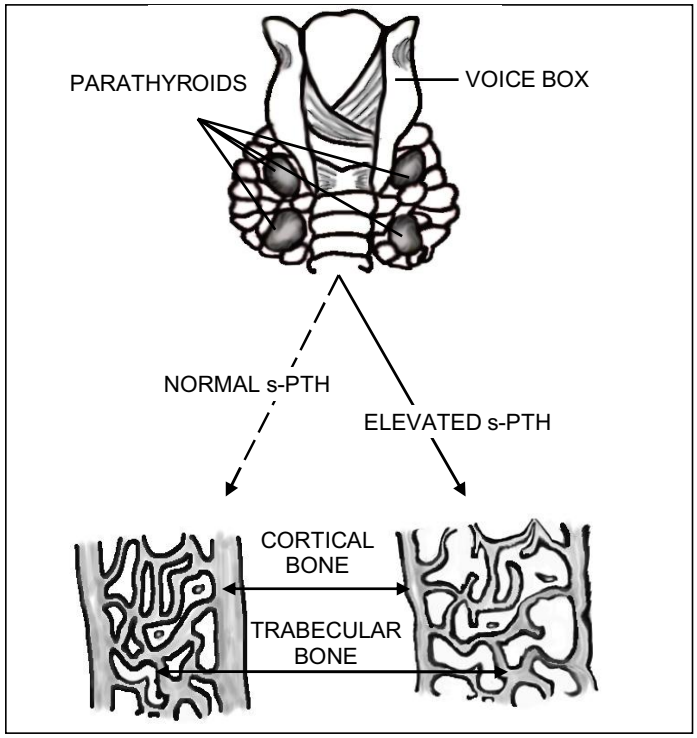

Figure 7: PTH has anabolic and catabolic effects on bone depending on the exposure. Continuously high s-PTH leads to increased turnover and bone destruction.

Bellido et al. (2003) have a mechanistic explanation for the opposite effects of PTH when administered intermittently and continuously. PTH acting through PKA prevents osteoblast apoptosis by inactivating proapoptotic Bad and transcription of survival genes, like Bcl-2. The transcription factors of cAMP response element-binding protein (CREB) and Cbfa1/Runx2 mediate the survival gene transcription. However, PTH also promotes the degradation of Cbfa1/Runx2. Continuous exposure to PTH leads to decreased level of Cbfa1/Runx2. This provides a negative feedback loop to diminish the anti-apoptotic effects of PTH. As PTH exposure continues, anti-apoptotic signals fade and osteoclastogenetic signals arise. Thus, repeated brief exposures to PTH promote osteoblast accumulation, but continued exposure tips the balance the other way and osteoclasts gain the upper hand (Figure 7).

2.4.3 PTH and Wnt signalling pathway

PTH regulates the expression levels of components of the Wnt pathway (Onyia et al. 2001), hence Wnt signalling might play a role in the bone response to PTH. PTH induces the expression of Fz1 and downregulates Dkk1, the Wnt antagonist (Kulkarni et al. 2005). The downregulation of Dkk1 could lead to increased signalling through LRP5 and an anabolic action on bone (Kulkarni et al. 2005). This conclusion is supported by the finding that PTH increases β-catenin accumulation (Kulkarni et al. 2005). Furthermore, in addition to promoting osteoblastogenesis, intermittent PTH inhibits adipogenesis (Rickard et al. 2006). This finding gives support to the involvement of the Wnt pathway since osteogenic and adipogenic differentiation from MSC have an inverse relationship, and the Wnt pathway inhibits adipogenesis. However, the effect of PTH on Wnt and LRP5 signalling is not clear, as a study with $LRP5^{-/-}$ mice shows intermittent treatment with PTH to increase cortical thickness (Sawakami et al. 2006). Hence, PTH does not require LRP5, but functions via a different signalling pathway (Sawakami et al. 2006).

3 AIMS OF THE STUDY

This study aimed to shed light on the mechanisms of high dietary phosphate intake on bone and to examine the influence of three bioactive tripeptides on osteoblast function and gene expression. The in vivo results contribute to a greater understanding of the role of dietary phosphate in bone health and the site-dependent effect of PTH. The in vitro results help explain how bioactive peptides affect osteoblasts and human mesenchymal stem cells and elucidate the mechanisms by which dietary protein supports bone health at the cellular level.

Specific aims were as follows:

1) To clarify the effect of a modest but prolonged increase in dietary intake of inorganic phosphate on bone quantitative factors in mature male rats (Study I).

2) To investigate the influence of high dietary phosphate intake on bone homeostasis and structure and on bone cells in intact growing male rats (Study II).

3) To specify the effects of IPP, VPP and LKP tripeptides on osteoblast proliferation in vitro and to clarify their function on gene expression (Study III).

4) To identify the long-term effect of IPP tripeptide on osteoblast gene-expression and differentiation by using the qPCR method (Study IV).

4 MATERIALS AND METHODS

Materials and methods are described briefly here. The detailed descriptions can be found in the original publications (I-IV).

4.1 Experimental design for in vivo studies (Studies I and II)
Male Wistar rats (HsdBrlHan:WIST) were housed in groups of two, three, or five in plastic cages at 21°C, 60 ± 10% humidity, and with a 12-h light/dark cycle. Access to water was ad libitum. Rats were offered 20 g of pellets daily of one of the diets varying in calcium:phosphate ratios. The control diet contained 0.6% phosphate (Ca:P 1:1), and other two diets, Ca:P 1:2 and Ca:P 1:3, contained 1.2% phosphate and 1.8% phosphate, respectively. The calcium content in all diets remained constant at 0.6%. Rats continued with the diets for 8 weeks. Detailed diet compositions are shown in original publications I and II. All diets were provided by the same supplier (Harlan Teklad, Madison, WI, USA). All study protocols were approved by the Ethics Committee for Animal Experimentation at the University of Helsinki.

4.1.1 Assessment of bone measurements

4.1.1.1 Dual-energy X-ray bone densitometry (DXA)

In Study I, we measured right femur and lumbar spine (L4-6) and in Study II right femur bone area (cm^2), bone mineral content (BMC, g) and bone mineral density (BMD, g/cm^2) in vivo with a DXA device (Lunar PIXImus^{TM}, GE Medical Systems, WI, USA) at onset as well as at the end of the 8-week study period.

4.1.1.2 Bone mineral content

In Study I, tibia calcium and phosphate contents were analysed with an inductively coupled plasma-mass spectrometer (ICP/MS, Perkin-Elmer Sciex Elan 6000, USA) using Quantitative Analysis techniques.

4.1.1.3 Peripheral quantitative computed tomography (pQCT)

In Study II, tomographic measurements of the right femurs were performed with pQCT (XCT540 Stratec; Norland Medical Systems, Pforzheim, Germany), and Stratec Software version 5.40 was used to analyse the images at the distal metaphysis and femur midshaft.

4.1.1.4 Microtomography (micro-CT)

Six tibias were scanned in a micro-CT system (Skyscan 1172, Skyscan N.V., Aartlesaar, Belgium) to obtain the three-dimensional bone structure of cortical and trabecular bone.

4.1.1.5 Mechanical testing

Right femurs and tibias were mechanically tested. The failure load of the femoral neck and the three-point bending strength of the tibial shaft were measured according to Peng et al. (1994, 1999) with a materials testing machine (1994). Stress and strain were calculated at yield, maximal and fracture points in the testing curves according to Turner & Burr (1993).

Following the bending test, horizontal and vertical (testing force direction) diameters were measured with a micrometer at the outer (A, B) and inner (a, b) parts of the tibia fracture site. The cross-sectional moment of inertia (I) was calculated at the fracture site as an ellipse, $I = \pi/64(AB^3-ab^3)$ (Bak & Jensen 1992).

4.1.2 Histomorphometric analyses

In Study I, the femur was fixed for 24 h with 10% formalin (Formal-Fixx™, Thermo Shandon, UK), decalcified (TBD-2™, Thermo Shandon, UK), dehydrated in graded ethanols and acetone and then embedded in paraffin. Three 5-μm-thick paraffin-embedded horizontal bone sections were cut from the proximal end of the diaphysis and dyed with a haematoxylin-eosin stain. Bone diameter, inner bone diameter and cortical width of the sections were measured using a 2x objective and a Nikon micrometer on a Nikon Eclipse E400 microscope. In addition to these measurements, the section modulus was calculated.

In Study II, bone labelling with an intraperitoneal injection of tetracycline (15 mg/kg, Oxytetracyclin, Pfizer) was performed 12 and 2 days prior to sacrifice.
For tissue collection, both hind legs were disarticulated at the hip, knee and ankle. The length of the right femur was measured. The samples were dehydrated in graded concentrations of ethanol and embedded undecalcified in methylmethacrylate. Longitudinal frontal sections of the distal femur were cut at a thickness of 7 μm using a Polycut S heavy-duty microtome (Leica Instruments GmbH, Heidelberg, Germany). The mineral apposition rate (MAR, μm/day) was measured in these sections by double tetracycline labelling using an MCID/M4 image analyser (Imaging Research Inc., Canada) and a Sony DXC 930P camera (Japan). The sections were stained with the Masson-Goldner-Trichrome method for studying structural properties. Histomorphometry was performed with a semiautomatic image analysing system linked to a light microscope (Nikon Optihot II, Japan). Trabecular bone area (Tb.Ar, %), total trabecular perimeter (B.Pm, mm), trabecular width (Tb.Wi, μm) and osteoblast perimeter (Ob.Pm, mm) were measured, and osteoclast number (Oc.N) was calculated.

4.1.3 Assessment of blood chemistry

At the end of the study, blood samples were drawn by cardiac puncture for serum parathyroid hormone (s-PTH) assessment with a Rat-intact PTH ELISA Kit (Immutopics, Inc., San Clementine, CA, USA). In Study II, serum calcium and phosphate concentrations were assessed with an automatic analyser (Konelab 20, Thermo Clinical Labsystems Oy, Espoo, Finland).

4.2 Experimental design for in vitro experiments (Studies III and IV)

4.2.1 Cell cultures

UMR-106 rat osteosarcoma cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS, 50 IU penicillin and 50 μg/ml streptomycin at 37°C in a humified atmosphere with 5% CO_2 on 96–

well plates. The cells were seeded at 1 x 10^4 cells/cm², cultured for 24 h, and the medium was then changed to a fresh medium containing various concentrations of IPP, VPP or LKP.

Human mesenchymal stem cells (hMSC, Poietics, Cambrex Bio Science, Walkerville, Maryland, USA) purified from human bone marrow were seeded at 3100 cells/cm² and cultured in Poietics mesenchymal stem cell growth medium with mesenchymal cell growth supplements (Cambrex Bio Science) at 37°C in a humified atmosphere with 5% CO_2. At 50% confluence the medium was substituted either by a fresh growth medium (α-MEM, 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin) or differentiation medium (α-MEM, 10% FBS, 10^{-8} M Dex, 50 µg/ml L-ascorbic acid, and 10 mM β–GP, 100 U/ml penicillin, 100 U/ml streptomycin). After 12-14 days of differentiation the osteoblastic phenotype was confirmed by amplifying osteocalcin with qRT-PCR, by alkaline phosphatase staining (Sigma histological alkaline phosphatase kit no. 86) and by assessing mineralization with Alizarin Red S staining (Bodine et al. 1996).

4.2.2 Tripeptides IPP, VPP and LKP

Peptides were synthesized in the Core Facility for Synthetic Peptides (Division of Biochemistry, Department of Biological and Environmental Sciences), University of Helsinki, Finland) by 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and purified by HPLC reverse-phase columns.

4.2.3 Analytical methods for in vitro experiments

4.2.3.1 Microarray analysis

Total RNA from the hMSC differentiated osteoblasts, and IPP-treated osteoblasts was extracted using a RNeasy Protect mini-kit (Qiagen GmgH, Hilden, Germany). The RNA concentration was measured at 260 nm by a spectrophotometer (SmartSpec™3000 Biorad). The cDNA microarray hybridizations were performed at the Finnish DNA Microarray Centre, University of Turku. The expression profiles of osteoblasts differentiated from hMSC were compared after a 24-h treatment with 50 µM IPP, VPP or LKP with a Hum16-K protocol, consisting of 16 000 human gene probes. Microarray data were processed with ScanArray and QuantArray. LOWESS normalizations, t-test and gene-expression profiles were analysed by Kensington Discovery Edition 2.0 software and TIGR Multi Experiment Viewer (MeV). A p-value of 0.05 was used as a limit for statistically significant differences in expression. Data were then further filtered according to fold difference in expression. At least a 1.8 fold difference was used in control/treatment comparison.

4.2.3.2 Quantitative RT-PCR

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed in a real-time quantitative PCR (Mx3000P, Stratagene) according to the manufacturer's instructions using a Brilliant SYBRGreen QPCR Master mix kit (Stratagene) and cDNAs as templates. Fluorescence data were collected during the annealing step and analysed with Mx3000P software. A dilution series of cDNA from human brain total RNA (BD,

Biosciences, Clontech) was used as a calibration standard, and the data were normalized by amplification result of ß-actin. All reactions were run in triplicate.

In study III, we used the same cDNA as in the microarray analysis (control and 50 µM, 24-h IPP-treated). The mean value was used to calculate the ratio of target gene/β-actin expression in each sample. Using the ratio in the untreated sample as a standard (100), the relative ratio of the treated sample was presented as the relative expression level of the target gene.

In study IV, the qPCR analysis was carried out on days 13, 17 and 20 of culture on hMSC-differentiated osteoblasts and osteoblasts treated with 50 µM IPP; hence, the results represent later stages of osteoblast differentiation. All qPCR primers are shown in Table 1.

Table 1: Nucleotide sequences of primers used for quantitative RT-PCR detection.

Gene	Primer sequence	Product size (bp)	Reference
β-actin	F: AGG CCA ACC GCG AGA AGA TGA CC R: GAA GTC CAG GGC GAC GTA GCAC	350	Wordinger et al. 2002
β-catenin	F: TTC TGG TGC CAC TAC CAC AGC R: TGC ATG CCC TCA TCT AAT GTC	216	Wang et al. 2000
BMP-2	F: GGA GAA GGA GGA GGC AAG R: GAC ACG TCC ATT GAA AGA GC	100	Emmanuele et al. 2003
BMP-5	F: AAG AGG ACA AGA AGG ACT AAA AAT AT R: GTA GAG ATC CAG CAT AAA GAG AGGT	303	Wordinger et al. 2002
Caspase-8	F: AGG AGG AGA TGG AAA GGG AAC TT R: ACC TCA ATT CTG ATC TGC TCA CTT CT	108	Gomez et al. 2003
Cbfa1/Runx2	F: TGA GAG CCG CTT CTC CAA CC R: GCG GAA GCA TTC TGG AAG GA	266	Primer3
CREB-5	F: GCT TTG GTG CTT TTC TCC AG R: GGT GAC ACC ACA GCA CAA AC	245	Primer3
OPG	F: GGC AAC ACA GCT CAC AAG AA R: CTG GGT TTG CAT GCC TTT AT	241	Kusumi et al. 2005
OCN	F: ATG AGA GCC CTC ACA CTC CTCG R: GTC AGC CAA CTC GTC ACA GTCC	255	Kuliwaba et al. 2000
Osteoglycin	F: TTG ATG CTG TAC CAC CCT TAC R: ATT CCA GGG CAT TAT GGT CC	348	Primer3
RANKL	F: AGA GCG CAG ATG GAT CCT AA R: TTC CTT TTG CAC AGC TCC TT	180	Primer3
PTHrP	F: GTC TCA GCC GCC GCC TCA A R: GGA AGA ATC GTC GCC GTA AA	93	Richard et al. 2003
VDR	F: CCA GTT CGT GTG AAT GAT GG R: CCT TTT GGA TGC TGT AAC TG	256	Primer3

All primer sequences used were designed as described previously, except primer pairs for Cbfa1/Runx2, CREB-5, osteoglycin, RANKL and VDR, which were designed with Primer 3 –software for this study.

4.2.3.3 Assessment of cell proliferation

The proliferation experiment was carried out with 5, 50 and 500 μM peptide concentrations and 6-, 24- or 48-h treatment time. DNA synthesis activity was assessed by a Cell Proliferation ELISA kit (Roche Diagnostics GmbH, Germany). The 5-bromo-2'-deoxyuridine (BrdU) incorporation was measured with a spectroscopic plate reader at 450 nm (Multiskan Ex, Thermo Labsystems, Helsinki, Finland).

4.2.3.4 Assessment of total protein content

Total protein content was assessed from cells grown on the 24-well plates according to the Bradford method (Bradford 1976) on days 3, 7, 12, 14, 17, 21 and 34 of culture. Bovine serum albumin (Sigma) was used as a standard.

4.2.3.5 Assessment of bone alkaline phosphatase activity

Bone-specific alkaline phosphatase (bALP) activity was measured at seven time-points with an OCTEIA™ Ostase® BAP immunoenzymetric assay (Immunodiagnostic Systems Limited, Boldon, UK) from cells grown on the 24-well plates. Prior to measurement, cells were washed with phosphate-buffered saline (PBS) and frozen (-70°C) with 300 μl of Tris –Triton (0.1 M Tris –base, 0.2% Triton X-1000). After thawing, the cells were centrifuged (12000 g, 5 min). Supernatant was used for the analysis. The ALP activity was corrected with the protein content according to the Bradford method (Bradford 1976).

4.2.3.6 Assessment of mineralization

Cell culture mineralization was assessed by Alizarin Red S staining from cells grown on the 96-well plates (Bodine et al. 1996). After staining, the dye was eluated with a solution of 10% cetylpyridinium chloride and 10 mM sodium phosphate (pH 7.0, 15 min, RT). Absorbance was measured at 590 nm (Multiskan Ex, Thermo Labsystems, Helsinki, Finland).

4.3 Statistical analyses

In Study I, comparisons between groups for BMD were tested by analysis of covariance (ANCOVA) with baseline bone mineral values and changes in body weight were used as covariates. Paired samples t-test was used to analyse changes within a group over the study period. Independent samples t-test was used to compare s-PTH concentration between groups. The correlation coefficient was tested by Pearson's correlation. Results were considered significant at a 95% significance level ($p<0.05$). Analyses were carried out using SPSS for Windows (version 12.0).

In Study II, we used one-way ANOVA, followed by Tukey's post-hoc test to determine the effects of diets or the least-significant difference (LSD) post-hoc test for pQCT values. For nonparametric values, the Kruskall-Wallis post-hoc test was used. Correlations between measured variables were tested for the whole group by Pearson's correlation, except for micro-CT, where Spearman's rho was used. Principal component analysis (PCA) was used to

analyse the histomorphometric measures and to determine the main components affected by a high-phosphate diet. Results were considered significant at 95% significance level ($p < 0.05$). The analyses were performed using SPSS for Windows (version 12.0).

In Studies III and IV, statistical analyses were performed with GraphPad Prism software (version 3.03) and SPSS for Windows (version 12.0). Data were analysed using either Student's unpaired t-test or ANOVA, followed by Tukey's post-hoc test. The microarray gene expression data for IPP-treated osteoblasts were analysed by PCA to categorize the affected genes. Results were considered significant at the 95% significance level ($p<0.05$).

5 RESULTS

5.1 In vivo Studies I and II

5.1.1 High phosphate intake reduced bone growth and mineral accrual (Study I and II)
In Study I, femur bone mineral density increased significantly in the control group, but not in the phosphate group when initial BMD and weight gain were used as covariates (Figure 8A). Lumbar (L4-6) BMD decreased in both groups (phosphate group -0.0026 ± 0.024 g/cm² and controls -0.0059 ± 0.024 g/cm²). The decrease in lumbar BMD was less in the phosphate group (Figure 8B).

In Study II, the Ca:P 1:3 rats had significantly shorter femurs than control or Ca:P 1:2 rats (p=0.001 and p=0.019, respectively). According to DXA results, final femur area values were not affected by a high phosphate intake. Both of the high-phosphate groups had significantly lower BMC and areal BMD than control rats (p<0.001 and p<0.001, respectively) (Figure BMD C). When the final DXA results were adjusted by femur length, the results remained unchanged. However, the DXA results must be interpreted with caution because rat body sizes differed between the groups. Weight-independent results from pQCT and micro-CT measurement are more reliable.

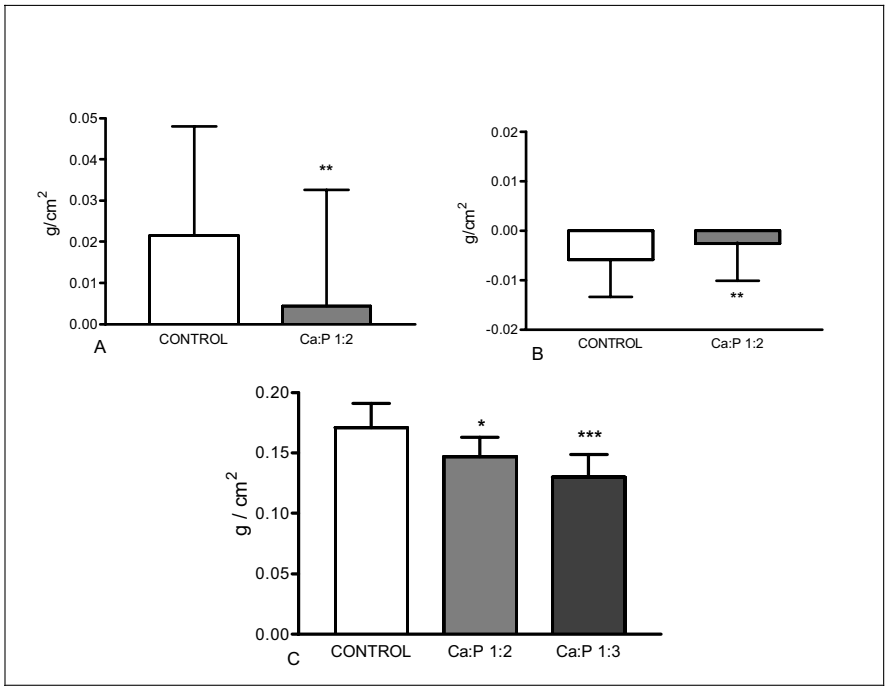

Figure 8: A) In aged rats (n = 20) femur ΔBMD was greater in control rats than in phosphate rats (p = 0.005). B) Spine BMD was reduced over the study period in aged rats. The reduction was greater in control rats (p = 0.007). C) In growing rats (n = 30) femur ΔBMD was reduced by high phosphate diet, (p < 0.001). Results are mean ± SD.

5.1.2 Tibia mineral content was unaffected by high phosphate diet (Study I)
No significant difference was found between groups in tibia calcium and phosphate content. Tibia BMC/body weight correlated with section modulus ($r=0.560$, $p=0.016$) as well as with calcium and phosphate content/bone length ($r=0.979$, $p<0.001$ and $r=0.974$, $p<0.001$, respectively).

5.1.3 High phosphate intake attenuates cortical bone (Study II)
The femur pQCT measurement showed high phosphate diet to affect cortical bone more severily than trabecular bone. In the distal metaphysis a high-phosphate diet decreased cortical cross-sectional area (CSA). Cortical BMC was decreased dose-dependently. The endoperi–ratio, representing trabecular CSA/cortical CSA, was increased with the Ca:P 1:2 diet as compared with the other diets. This was a result of increased trabecular CSA in Ca:P 1:2 rats. However, total bone CSA (TB CSA) was reduced as was the total bone BMC (TB BMC).

In the femur midshaft, TB BMC and TB CSA decreased with increasing phosphate intake. Phosphate-rich diets did not affect trabecular bone, but decreased cortical BMC, CSA and thickness dose-dependently.

Our interpretation of the results is that periosteal and longitudinal growth were lower and endocortical resorption higher in the high-phosphate diet, leading to an absolute thinner cortex. Thus, the phosphate-rich diet restricted growth, resulting in short bones with a thin cortex and a lower absolute amount of bone. The volumetric total bone BMD (TB BMD) was reduced with a high-phosphate diet (control vs. Ca:P 1:3), hence there is less accrual in smaller bones. The volumetric TB BMD differs in the femur midshaft, but not in the distal metaphysis.However, the amount of trabecular bone (Trab CSA/Cort CSA) in the distal metaphysis differs between groups ($p=0.003$), whereas in the femur midshaft it does not. Bone metabolism is accelerated in the high-phosphate group, and this compensates some of the decrease in mineral accrual, but only in the distal metaphysis, as it has more trabecular bone. The femur midshaft has less trabecular bone, hence, the accelerated trabecular metabolism is not sufficient to compensate for the mineral loss in the cortical parts. Table 2 summarizes the high phosphate effects on bone measurements.

5.1.4 Bone microstructure was decreased due to high phosphate intake (Study II)
Micro-CT analysis showed a high-phosphate intake to decrease bone volume (BV) and bone surface (BS) values in trabecular bone. Trabecular separation (Tb.Sp) was largest in Ca:P 1:2 rats. The structure model index (SMI) was lower for Ca:P 1:3 rats, than for control or Ca:P 1:2 animals, indicating an increased plate/rod ratio in trabecular bone architecture. A positive trend was found in the Ca:P 1:3 diet in increasing the number of connective structures per unit volume (E.Conn.D).

In cortical bone, the total cross-sectional area (B.Ar) was decreased by a high phosphate intake. Cortical porosity (Po) had increased trend in high phosphate groups and a positive correlation with s-PTH. Cortical porosity and tibia ultimate strength had a negative correlation.

Table 2: Summary of the high dietary phosphate effect on bone measurements after 8 week feeding (↑ increase, ↓ decrease, ↔ no difference).

	Study I, adult rats Ca:P 1:2	Study II, growing rats Ca:P 1:2	Ca:P 1:3
Femur DXA			
BMD (g/ cm²)		↓	↓
BMC (g)	↔	↔	↓
Area (cm²)	↔	↔	↔
Lumbar DXA	↔		
BMD			
BMC	↔		
Area	↔		
Tibia Ca and P content	↔		
Femur length (cm)		↔	↓
pQCT of distal metaphysis			
TB BMD (g/cm^3)		↔	↔
TB BMC (g)		↓	↓
TB CSA (mm^2)		↓	↓
Trab BMD (g/cm^3)		↓	↔
Tra BMC (g)		↔	↔
Trab CSA (mm^2)		↑	↔
Cort BMD (g/cm^3)		↔	↔
Cort BMC (g)		↓	↓
Cort CSA (mm^2)		↓	↓
Mean Cort Th (mm)		↔	↔
Endoperi ratio		↑	↔
pQCT of femur midshaft			
TB BMD (g/cm^3)		↔	↓
TB BMC (g)		↓	↓
TB CSA (mm^2)		↓	↓
Trab BMD (g/cm^3)		↔	↔
Tra BMC (g)		↔	↔
Trab CSA (mm^2)		↔	↔
Cort BMD (g/cm^3)		↔	↓
Cort BMC (g)		↓	↓
Cort CSA (mm^2)		↓	↓
Mean Cort Th (mm)		↔	↓
Endoperi ratio		↔	↔
Section modulus		↔	↔

(continues)

(Table 2 continues)

	Ca:P 1:2	Ca:P 1:3
Tibia micro-CT		
End.Pm/Peri.Pm	↔	↔
MMI(polar) (mm^4)	↔	↔
Trabecular bone		
TV (mm^3)	↔	↔
BV (mm^3)	↓	↔
BS (mm^2)	↓	↔
Tb.Th (mm)	↔	↔
Tb.Sp (mm)	↑	↓
Tb.N (mm^{-1})	↔	↔
SMI	↔	↑
E.Conn.D (mm^{-3})	↔	↔
DA	↔	↔
Cortical bone		
B.Ar (mm^2)	↓	↓
Ct.Th (mm)	↔	↔
Po (%)	↔	↔
Mechanical competence of the femoral neck		
Ultimate strength (N)	↔	↓
Stiffness (N/mm)	↔	↔
Toughness (Nm x 10^{-3})	↔	↔
Yield point	↔	↓
Mechanical competence of tibia shaft		
Ultimate strength (N)	↓	↓
Stiffness (N/mm)	↔	↓
Toughness (Nm x 10^{-3})	↓	↓
Yield point	↔	↓

5.1.5 High phosphate intake reduced bone mechanical competence (Study II)
Dietary phosphate reduced femur shaft ultimate strength and yielding point dose-dependently. Ca:P 1:2 rats had 10% lower values for femur ultimate strength and 20% lower values for shaft yielding point than controls. The respective values for Ca:P 1:3 rats were 24% and 36% lower. PTH values had a negative correlation with femur ultimate strength, yielding point and toughness.

High phosphate intake reduced tibia mechanical competence. Values for Ca:P 1:2 tibias were 12% lower in ultimate strength, 11% lower in stiffness and 32% lower in toughness than for control tibias. In Ca:P 1:3 tibias, the corresponding values were 29%, 24% and 40%. Serum PTH correlated negatively with all of the above values.

In addition to decreased mechanical competence, tibia material properties were decreased. The stress-strain relationship is presented in Figure 9A. Stress values for yield, maximum

stress and fracture revealed that a high-phosphate diet reduces yield and maximum stress (Figure 9B).

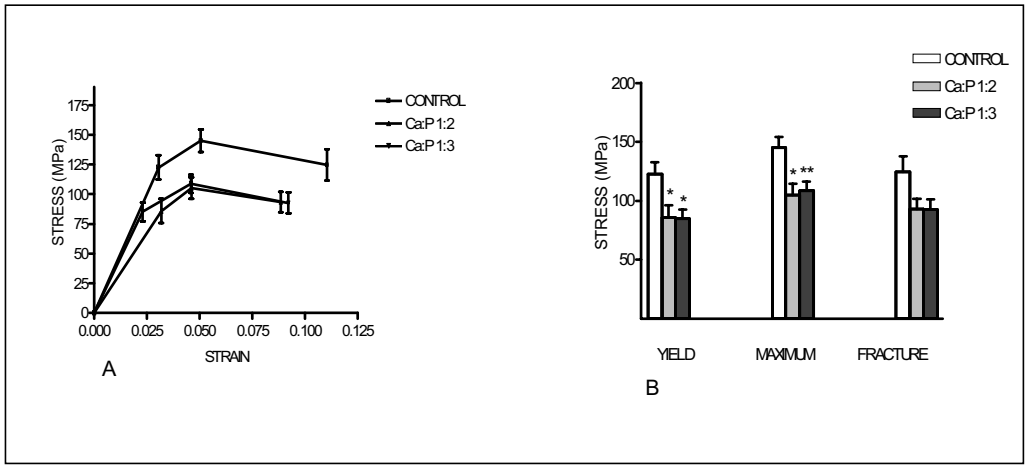

Figure 9: The stress-strain curve (A) and maximum stress values (B) for tibiae (n = 30). The high-phosphate diet reduced stress endurance, leading to lower yield and lower maximum stress values (* $p<0.05$, ** $p<0.01$). Results are mean ± SE.

5.1.6 Bone metabolim was increased by high phosphate intake (Study I and II)
In Study I, no significant differences emerged between groups in any of the measured morphometric parameters of the femur midshaft. Femur Δarea and spine Δarea had a negative correlation, and femur Δarea and femur cortex width had a positive correlation. Section modulus and periosteal and endosteal radius correlated with the following variables only within control animals: final femur area, femur Δarea and cortex. Values correlating negatively within the phosphate group only were lumbar spine Δarea with both femur diameter and marrow diameter.

In Study II, high phosphate intake reduced Tb.Ar and Tb.Wi dose-dependently and B.Pm, but increased Ob.Pm, Oc.N and MAR. S-PTH had a positive correlation with Oc.N and MAR, whereas final femur BMD had a negative correlation with Oc.N and MAR.

According to PCA, 71% of the variance in the six histomorphometric variables could be attributed to the first principal component. Because it had heavy positive loadings (>0.8) on Tb.Ar, Tb.Wi, and B.Pm. and heavy negative loadings (<-0.6) on Ob.Pm, Oc.N, and MAR, it was named ‘Bone volume’. The bone volume was highest in the control group, whereas high dietary phosphate intake resulted in lower bone volume.

5.1.7 High phosphate intake increased serum PTH (Studies I and II)
Serum PTH was significantly higher in the phosphate group at the end of the Study I (Figure 10A). In Study II, serum PTH was significantly higher in Ca:P 1:3 rats than in control or Ca:P 1:2 rats (Figure 10B). Serum calcium and phosphate did not differ between groups. Serum calcium had a positive correlation with final femur BMD. Serum phosphate had a positive correlation with s-PTH.

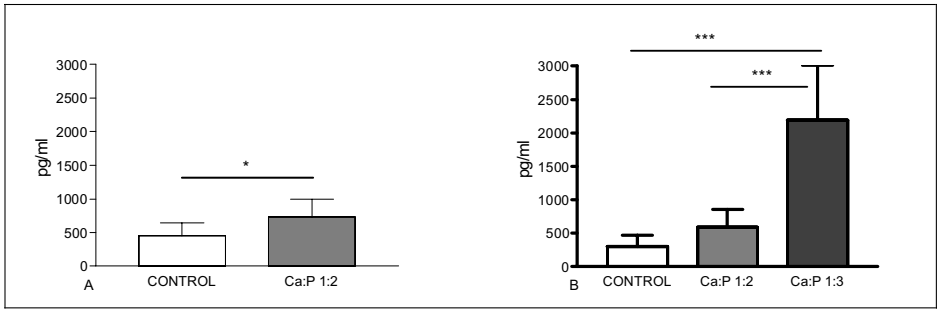

Figure 10: High-phosphate diet elevated serum PTH in the adult rats (n = 20) (A) and growing rats (n = 30) (B). *p < 0.05, ***p < 0.001. Results are mean ± SD.

5.1.8 Body weight gain and food intake (Studies I and II)
In Study I, the initial body weight, weight gain and final body weight did not differ between groups (Figure 11A). Body weight of rats in both groups significantly increased over the 8–week study period. Food intake was not recorded.

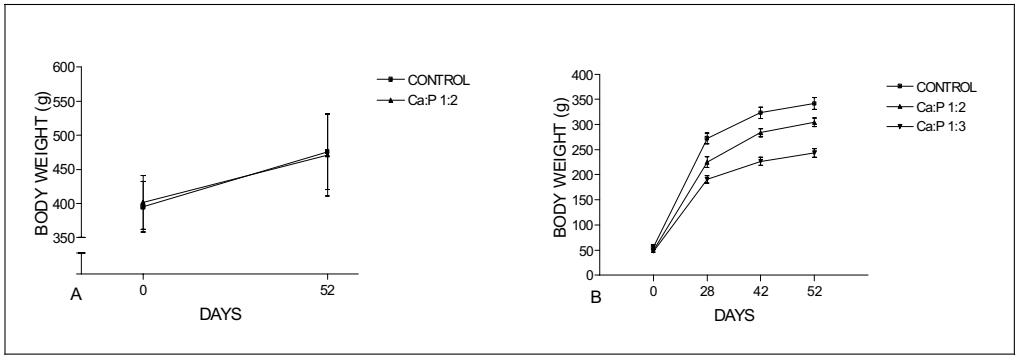

Figure 11: Weight evolution in aged rats (n 0 20) (A) and growing rats (n = 30) (B). Weight gain was significantly less in growing rats fed high phosphate diet (p < 0.001). Results are mean ± SD.

In Study II, a high-phosphate diet reduced growth. Rats in the Ca:P 1:2 group were 11% lighter and rats in the Ca:P 1:3 group 29% lighter than controls (Figure 11B). Due to lower body weight, the total food consumption was also less in high-phosphate groups (control, 10.3

kg; Ca:P 1:2, 9.9 kg; Ca:P 1:3, 9.2 kg). Adjusted food consumption (g of food/g of weight gain) varied between groups. In order to gain 1 g of weight, a rat needed to consume 2.5 g of control diet, 2.6 g of Ca:P 1:2 diet or 3.2 g of Ca:P 1:3 diet. The relative food consumption was 28% more in the Ca:P 1:3 group than in the control group.

To sum up the results of Studies I and II, an increased intake of dietary phosphate hindered mineral deposition into cortical bone and diminished BMD in aged rats. Phosphate elevated PTH and conserved trabecular bone, as mineral loss was lower in the lumbar spine with a phosphate-rich diet. In young rats, increased phosphate intake reduced bone material and structural properties and led to diminished bone strength. Our study shows a low Ca:P ratio to exhibit negative effects on mature and growing skeletons even when calcium intake is sufficient.

5.2 In vitro Studies III and IV

5.2.1 Microarray results from IPP-, VPP- or LKP-treated osteoblasts (Study III)
Microarray data analysis revealed IPP to regulate more genes in osteoblasts than VPP or LKP. IPP upregulated 270 genes and downregulated 100 genes. The respective numbers for VPP were 25 and 10 and for LKP 16 and 14. IPP not only regulated a larger number of genes than VPP or LKP, but IPP was the only tripeptide to upregulate osteogenic differentiation factors (see Study III, Table 2 for summary).

5.2.2 Quantitative RT-PCR results (Studies III and IV)
In Study III, the qRT-PCR results were in accordance with those of microarray analysis and showed IPP to increase CREB-5, BMP-5 and PTHrP expression, whereas VDR and caspase-8 expression were decreased. However, the effect of IPP on BMP-2 was not statistically significant in qRT-PCR analysis (see Study III, Figure 3).

In Study IV, the long-term effects of IPP on osteoblast gene expression are summarized in Table 3. More detailed figures are found in Study IV, Figure 3.

In short, long-term treatment with IPP increased osteoblast osteoglycin expression and reduced PTHrP and OPG expression on day 13. On day 17, IPP increased osteoblast β-catenin and osteoglycin expression, and reduced Cbfa1/Runx2, CREB-5 and Caspase-8 expression and RANKL/OPG ratio. On day 20, IPP reduced osteoblast PTHrP expression. Control cell PTHrP expression was highest on day 13, reduced on day 17 and elevated again on day 20. IPP inhibited the rise on day 13 and on day 20. Control cell osteoglycin expression did not differ between days 13, 17, and 20. IPP increased osteoglycin expression on day 13 and day 17. Caspase-8 was elevated in control cells from day 13 to day 17 and reduced from day 17 to day 20. IPP suppressed the day 17 elevation in caspase-8 expression.

OPG expression did not differ in control cells at day 13, 17, or 20. IPP reduced OPG expression at day 13. No significant changes occurred in RANKL expression over the study

period in control or IPP treated cells. RANKL was present in very low concentrations, and despite four separate RT-PCR analyses the large standard deviation masked potential difference. The RANKL/OPG ratio was increased in control cells from day 13 onwards. IPP reduced the RANKL/OPG ratio at day 17.

Table 3: Summary of the RT-qPCR results from long-term treatment with IPP (50 µM).

	IPP 50 µM		
	Day 13	**Day 17**	**Day 20**
β-catenin		↑	
Cbfa1/Runx2		↓	
PTHrP	↓		↓
CREB-5		↓	
Osteoglycin	↑	↑	
Osteocalcin			
Caspase-8		↓	
OPG	↓		
RANKL			
RANKL/OPG		↓	

5.2.3 Cell proliferation (Study III)
IPP, VPP and LKP increased UMR-106 cell proliferation as compared with that of control cells. A 24-h treatment with 50 µM IPP increased hMSC cell proliferation. In a dose-response study with mature osteoblasts IPP decreased the proliferation at a concentration of 500 µM. There was no time-response on osteoblast proliferation with 50 µM IPP at 3, 24 or 48 h (Figure 12).

5.2.4 Total protein content (Study IV)
Total protein content increased in all groups from day 3 to day 34 (Figure 13A). Total protein content did not differ between groups.

5.2.5 Alkaline phosphatase activity (Study IV)
Bone alkaline phosphatase (bALP) activity increased from day 7 onwards in control and IPP-treated cells grown in an osteoblast differentiation medium, but not in cells grown in amesenchymal cell growth medium (Figure 13B). bALP activity further increased up to day 21 and remained high until day 34. bALP activity did not differ between IPP-treated osteoblasts and control cells.

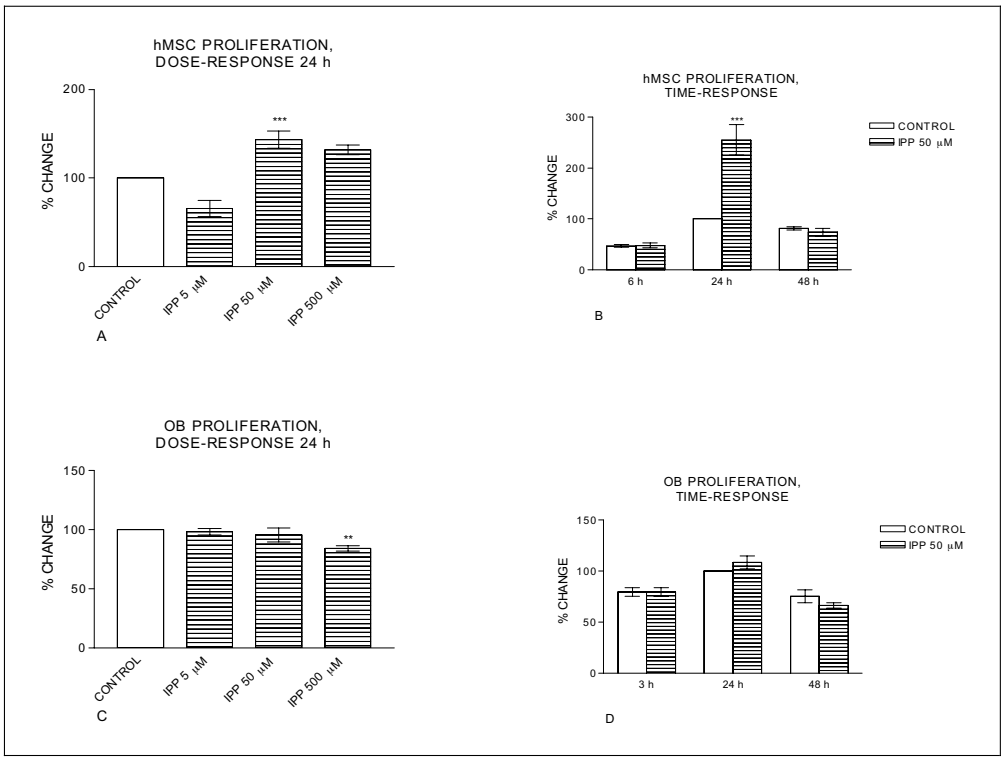

Figure 12: The effect of IPP tripeptide on cell proliferation in hMSC (A and B) and differentiated osteoblasts (OB) (C and D). Dose- and time-response. The results are shown as the relative amounts of BrdU incorporation. The mean amount of BrdU incorporation in the 24 h control cells has been given the value 100 (** $p<0.01$, *** $p<0.001$). Results are mean ± SD (n = 5).

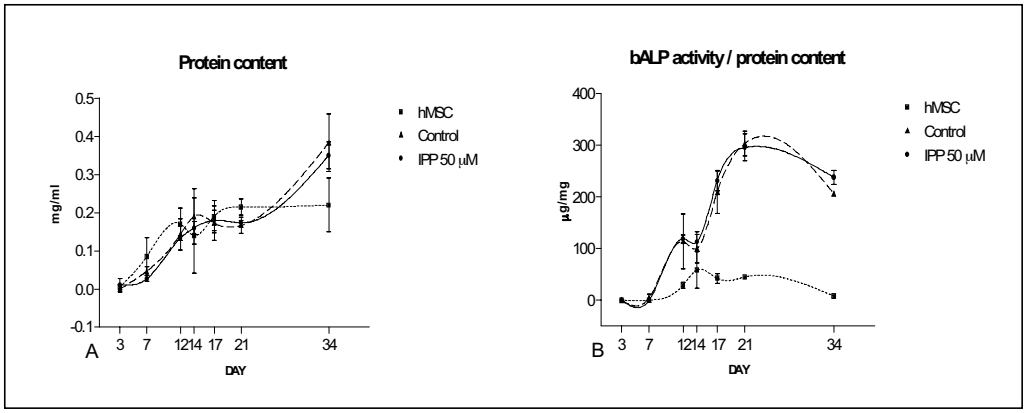

Figure 13: Protein content did not differ between cell cultures (A). bALP activity was increased in cells grown with osteoblast differentiation medium (B). IPP had no effect on bALP activity. Results are mean ± SD (n = 3).

5.2.6 Mineralization (Study IV)
IPP (50 μM) treatment increased osteoblast mineralization (Figure 14).

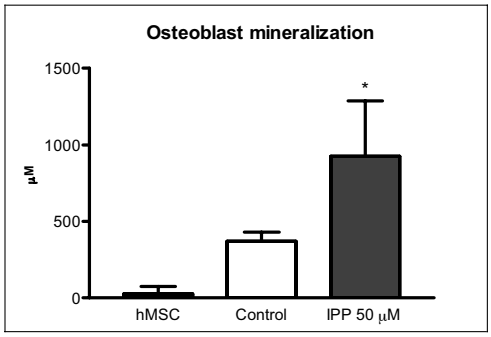

Figure 14:
Continuous exposure to 50 μM IPP increased osteoblast mineralization (n = 6). Results are mean ± SD. *p < 0.05

To sum up findings of Studies III and IV, short-term treatment with tripeptide IPP, VPP or LKP had an effect on osteoblast gene expression. IPP in particular, was efficient in regulating genes associated with cell differentiation, cell growth and cell signal transcription. The upregulation of these genes indicated that IPP enhances osteoblast proliferation and differentiation. IPP was confirmed to increase UMR-106 and hMSC proliferation, but not the proliferation of mature osteoblasts. Long-term treatment with tripeptide IPP enhanced osteoblast gene expression in favour of bone formation and increased mineralization. Long-term treatment with IPP did not influence osteoblast differentiation in vitro. However, the in vivo effects might differ, since eating frequency drives food consumption, and protein degradation products, such as bioactive peptides, are available periodically, not continuously as in this study.

6 DISCUSSION

Bone health is influenced by diet. Nutrients important for bone growth should therefore be recommended, but bone-impairing nutrients must also be noted (Ward et al. 2003). In the daily diet, nutrients are consumed in conjunction foods, and associations attributed to a single nutrient may in fact be caused by the simultaneous consumption of other nutrients (Tucker et al. 2002). To study the effect of a single nutrient on bone, structured trials are needed. This thesis examines the in vivo effects of inorganic phosphate and the in vitro effects of bioactive tripeptides on bone. Studies I and II are based on a rodent model and Studies III and IV on cell culture models.

Studies I and II revealed high intake of inorganic phosphate to have deleterious effects on bone. High phosphate diet restiricted mineral deposition into cortical bone and resulted to lower bone mineral accrual in femurs in mature rats (Study I). In order to maintain strength bones compensated the lack of mineral by an increase in size. Bone loss is part of normal aging. The rate of bone loss is greater in trabecular than in cortical bone (O'Flaherty 2000). Vertebrae is mainly composed of trabecular bone (Riggs et al. 1981). All aged rats had a reduction in lumbar mineral content over the 8-week study, but the decrease was less in the high phosphate group. Similar difference between cortical and trabecular bone loss was also seen in growing rats (Study II). The pQCT results revealed high phosphate diet to reduce cortical thickness and mineral content dose-dependently, whereas trabecular bone was less affected. Total bone mineral density was reduced only at areas rich in cortical bone but elsewhere trabecular bone subsidised for mineral loss. In the growing rats high phosphate diet did not only reduce mineral accrual into cortical bone but also hindered the growth of the animals. The growth retard was a result of the diet. Something about the high phosphate diet restrained the animals from growing, in order to gain 1 g of weight an animal had to eat 28% more of the high phosphate diet than control diet. Smaller rats in the high-phosphate groups accordingly had smaller bones. Although the total bone length was reduced by high phosphate diet, the relative width of the long bones was actually increased, as no differences were present between control and high-phosphate groups in DXA-measured femur areas, pQCT-measured femur diameter trabecular bone cross-sectional area, or micrometer-measured tibia fracture-site diameters between groups. Hence, we conclude that high-phosphate diet reduced cortical mineral content and affected bone geometry.

Further histological investigation of the bones from the Study II revealed high phosphate diet to increase bone remodelling as the number of bone-resorbing osteoclasts and bone-forming osteoblasts was increased. Bone remodelling at a normal rate is a necessity for the maintenance of bone but remodelling beyond appropriate levels results in diminished structure. The structural deterioration of the tibias was seen in micro-CT results as high phosphate diet decreased trabecular bone volume and surface, increased trabecular separation and reduced cortical bone cross-sectional area. The structural and material changes caused by the high-phosphate diet weakened total bone strength and reduced the stress-strain endurance

leading to lower ultimate bone strength. Alterations in bone geometry could not overcome these obstacles.

Many of the deleterious effects of high-phosphate diet, such as the site-dependent action on cortical and trabecular bone, could be mediated by PTH (Freesmeyer et al. 2001). Serum concentrations of PTH rise with age in rats and humans (Fox & Mathew 1991, Halloran et al. 2002). Also high dietary phosphorus intake increases s-PTH (Almaden et al, 1996, Almaden et al. 1998, Kärkkäinen & Lamberg-Allardt 1996, Miyamoto et al. 1998, Koshihara 2001). Continuously elevated PTH has catabolic effects on bone as it decreases bone mass by stimulating bone resorption (Ma et al. 2001, Halloran et al. 2002). In addition to reduced bone mineral content elevated serum PTH impairs bone structure by inducing cortical porosity (Lotinun et al. 2004, Tazawa et al. 2004). These events are brought about by PTH's effects on bone cells. PTH directly stimulates osteoclast activity (Dempster et al. 2005) and indirectly, via osteoblasts, increases osteoclastogenesis (Locklin et al. 2003, Ma et al. 2001). PTH increases osteoblast RANKL expression and decreases OPG expression (Ma et al. 2001). The increase in RANKL/OPG ratio leads to osteoclastogenesis and bone loss. In addition, PTH inhibits osteoblast collagen synthesis and thus bone-formation (Canalis et al. 1989, Canalis et al. 1990). As bone toughness is determined by collagen (Currey 2003), changes in collagen alter the mechanical properties of bone and affect bone strength (Burr 2002). In addition to bone cells, PTH also acts on chondrocytes. PTH delays chondrocyte hypertrophy (Guo et al. 2006), and downregulates their terminal differentiation and collagen expression (Harrington et al. 2004). In our Study II the elevated PTH could have affected chondrocyte differentiation and function in the growth plates and hence impaired the growth of the animals. Elevated PTH impairs bone in many ways and the elevated s-PTH levels, seen in adult and growing rats, might well have contributed to the diminished bone strength. However, the growing rats on Ca:P 1:2 diet (Study II) did not have a significant rise in s-PTH compared with controls, although many of the measured parameters did decline, indicating that high dietary phosphate has a bone weakening effect beyond PTH.

Bone strength is a combination of several factors; geometric, structural and material properties (Peng et al. 1994). Because bone is a metabolically active tissue equipped with a mechanostat (bone mass and strength are regulated to meet mechanical demands, for review, see Skerry 2006), a decrease in one factor leads to compensation by an other to maintain the overall bone strength. In our studies the rats in high-phosphate groups had less mineral deposited into femurs than control rats, and this was compensated by an increase in bone size. Bone size directly reflects bone strength; bigger bones have higher absolute strength (Orwoll 2003), but small bones can have similar relative strength if the material properties are equal. Mineral content is the most important material property affecting bone stiffness and ultimate strength (Seeman 2003). The mechanical properties of bone vary at different structural levels (Rho et al. 1998) and in trabecular bone, the overall structure is even more important than mineral content (Currey 2003). Our results show, that a high-phosphate diet reduces bone mineral accrual into cortical bone and increases bone remodelling beyond appropriate levels,

resulting in diminished trabecular bone structure. In the future it would be interesting to further characterize the effects of high dietary phosphate on osteoblast function in vivo as our results revealed negative correlation with s-PTH and bone toughness, which could be a result of decreased collagen in bone matrix. Bone mineral-collagen-ratio could be analysed with a method such as fourier transform infrared imaging spectroscopy (FT-IRIS).

These studies (I and II) were carried out in rats, as rodent models are commonly used in bone research. The constraints faced by such a translational study are the continuous longitudinal growth of bone accompanied by periosteal modelling, the lack of a Haversian system in rats and the sensitivity of rats to dietary phosphate (Ritskes-Hoitinga et al. 1993, Bagi et al. 1997). Study II described the effect of high dietary phosphate on the growing skeleton, hence, the first two obstacles were overcome, as they are also present in the growing human skeleton. The lack of a Haversian system in rats can attenuate cortical porosity (Dempster 1993). In humans, a high-phosphate diet could enhance cortical porosity because of active Haversian remodelling compared with the rat. To overcome the obstacle of rats being phosphate-sensitive, we used male rats, which are reported to be more resistant than females to dietary phosphate (Ritskes-Hoitinga et al. 1993).

Based on these long-term dietary intervention results from animal studies and previous short-term interventions on humans, it is very likely that a long-term human trial would yield negative outcome on bones. Short-term high-phosphate and low-calcium interventions on human subjects have shown elevated s-PTH (Calvo et al. 1990, Brixen et al. 1992, Kärkkäinen & Lamberg-Allardt 1996), decreased bone formation marker (bALP) (Kärkkäinen & Lamberg-Allardt 1996) and increased bone resorption markers (S-CTX and U-NTX) (Kristensen et al. 2005). Besides not being absolutely necessary, a long-term high-phosphate intervention on humans would be unethical, although it would give the ultimate answer to speculation about the effect of phosphates on the human skeleton. The results from our Studies I and II indicate high dietary phosphate to have deleterious effects on rat skeleton, which could not be overcome by adequate calcium intake. As it is widely accepted that adequate calcium intake can revert the adverse effects of e.g. high-dietary phosphorus intake (NIH consensus conference 2001), our results should raise concerns when deciding on sufficient calcium and phosphate intake to support bone health.

Our in vitro studies (III and IV) were carried out to investigate the effects of bioactive tripeptides on human osteoblasts. Bioactive peptides are derived from dietary protein. Epidemiological data shows dietary protein to have positive effects on bone metabolism (e.g. Hannan et al. 2000, Hoppe et al. 2000, Kerstetter et al. 2003). We hypothesized that bioactive peptides would have positive effects on bone as they are produced in hydrolysis of proteins and have a variety of biological functions in body. We studied the effects of IPP, VPP and LKP as they are all common food derived peptides, have a structure that is suited for biological function in addition to tissue building and all possess ACE-inhibitory potential. A preliminary screening of the tripeptides IPP, VPP and LKP on osteoblast proliferation was

carried out on UMR-106 cells. The rat osteosarcoma cell-line UMR-106 is commonly used as a model of the osteoblast phenotype (e.g. Hickman & McElduff 1989, Mitchell et al. 1990). However, the in vitro studies performed using osteoblasts differentiated from human mesenchymal stem cells yield more applicable information on human osteoblast function since the osteosarcoma cell line or mouse osteoblasts might not correctly reflect human osteoblast function (Shui et al. 2003). Hence, further studies were carried out on hMSC-differentiated osteoblasts. Study III revealed that all of the tripeptides studied, IPP, VPP and LKP, increased cell proliferation and induced osteoblast gene-expression. Based on the proliferation results 50 μM peptide concentration and 24-h treatment-time were used to induce hMSCs to differentiate towards osteoblasts before performing microarray analysis. The results show IPP to be the most effective in regulating osteoblast gene expression in respect to VPP and LKP. IPP upregulated over 6.5 times more genes and downregulated 4 times more genes than VPP and LKP together. Furthermore, IPP was the only tripeptide to upregulate osteogenic factors. We therefore further investigated IPP's short- and long-term effects on osteoblast differentiation. The short-term effects (Study III) indicated that IPP increases cell differentiation, growth, transcription and viability. In short-term treatment with IPP, PTHrP could play a role in mediating these functions, as IPP increased osteoblast PTHrP mRNA expression. PTHrP produced by osteoblasts is thought to function locally within the skeletal microenvironment and to propel the bone marrow stromal cells towards the osteogenic lineage (Karaplis & Goltzman 2000). PTHrP also exerts anti-apoptotic effects (Karaplis & Goltzman 2000). IPP upregulated PTHrP and the transcription activator CREB-5, whereas apoptotic caspase-8 was downregulated. These findings indicate that bioactive peptide IPP can enhance gene expression on mRNA level in a way that increases the differentiation of hMSC into osteoblast lineage and prolongs cell viability. The osteogenic action could contribute to increased bone formation.

Long-term treatment with IPP (Study IV) showed enhanced osteoblast gene expression in favour of bone formation and increased mineralization. Continuous treatment with IPP does not seem to increase osteoblast differentiation in vitro when assessed by bALP activity. But since osteoblast differentiation is not regulated by a single set of factors, not all genes need to be expressed similarily in order to upregulate osteoblast markers (Liu et al. 2003, Osyczka & Leboy 2005). So eventhough there was no difference between bALP-activity, long-term treatment with IPP did increase matrix production and mineralization and reduced RANKL/OPG ratio. All of these factors would contribute to increased bone mass by enhancing bone-formation and reducing bone-resorption.

The positive osteogenic long-term effects of IPP seem to be mediated by Wnt/β-catenin pathway rather than PTHrP. Similarily, as PTHrP pushes cells towards the osteogenic lineage (Karaplis & Goltzman 2000), the Wnt/β-catenin pathway also stimulates osteoblastogenesis (Gaur et al. 2005). Furthermore, the Wnt/β-catenin pathway activates programs directing cell proliferation (Kohn & Moon 2005) and cell survival (Kohn & Moon 2005, Krishnan et al. 2006) and represses osteoclastogenesis (Spencer et al. 2006) and adipogenesis (Krishnan et al.

2006). Ageing is associated with a reciprocal increase in adipogenesis and a decrease in osteogenesis (Akune et al. 2004). Changing the adipogenic differentiation of MSCs to osteogenic differentiation will increase bone, as ‘fat’s loss is bone’s gain’ (Pei & Tontonoz 2004). IPPs potential to activate the Wnt/β-catenin pathway further supports our hypothesis of protein derived bioactive peptides to have positive effects on bone.

To summarize our findings from Studies III and IV, hMSC treatment with IPP favours osteoblastogenesis in vitro. The short- and long-term treatment mechanisms of action might vary. Bioactive peptides, such as IPP, may be one of the factors contributing to the positive skeletal effects that dietary protein has. In vivo IPP might have an even more marked effect. Peptides are produced from protein rich food at regular and frequent doses which could enhance their effect. Frequent small meals are known to increase bone quality (Li & Muhlbauer 1999). Further studies are, however, needed to clarify the in vivo mechanism of action and effective dose of bioactive tripeptides. It would be valuable also to know the amount of different active peptides being produced from different sources of protein and their interactions in vivo.

7 CONCLUSIONS

Results of our in vivo studies (I and II) indicate that long-term high dietary phosphorus intake has an unfavourable effect on a rodent bone. In adult male rats, high dietary intake of inorganic phosphate reduced cortical BMD but protected subjects from trabecular bone loss. This site-dependent effect could, at least in part, be due to the increase in s-PTH. High-phosphate rats seemed to compensate for the lack of mineral deposition in femurs by an increase in cross-sectional size, as the phosphate-rich diet increased periosteal bone formation and resulted in wider bones. In growing male rats, high phosphate intake impaired animal growth, limited bone longitudinal growth and restricted femur BMC and BMD build-up. Bone structure was impaired, as reflected by decreased trabecular area and width and reduced mean cortical thickness. The changes in material and structural parameters resulted in diminished bone strength. Bone histomorphometry revealed increased bone metabolism since osteoclast number and osteoblast perimeter were increased, leading to an increased mineral apposition rate. In young rats, triplicating the phosphate intake elevated s-PTH. These results indicate that a low Ca:P ratio produces negative effects on the rat skeleton even with sufficient calcium intake.

Our in vitro studies (III and IV) on hMSC established that the bioactive tripeptides IPP, VKP and LKP increase cell proliferation. IPP, in particular, possesses osteogenic potential, as it upregulates genes associated with osteoblast differentiation, cell growth and cell transcription while apoptosis-related genes are downregulated. Short-term IPP treatment favours osteogenesis by propelling cells towards the osteogenic lineage. IPP's long-term effects on osteoblasts comprise increased matrix production and mineralization and a reduced RANKL/OPG ratio, which could lead to diminished osteoclastogenesis and resorption, further enhancing bone formation. Long-term treatment with IPP did not affect osteoblast differentiation when assessed by bone alkaline phosphatase activity. These results support the conclusion that bioactive peptides, such as IPP, have osteogenic potential and enhance osteoblast function in vitro.

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Helsinki, April 2007 Heini Huttunen

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